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PLATELET ANGIOGENIC ACTIVITIES AND THEIR REGULATION ON ENDOTHELIAL PROGENITOR CELL FUNCTIONS

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PLATELET ANGIOGENIC ACTIVITIES AND THEIR REGULATION ON ENDOTHELIAL PROGENITOR CELL FUNCTIONS

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ABSTRACT

Platelets are essential for haemostasis and thrombosis, but also play a major role in angiogenesis. Activated platelets recruit progenitor cells and induce differentiation into endothelial progenitor cells (EPCs), which contribute importantly to vascular regeneration and endothelium reparation. However, the interactions between platelets and EPCs are poorly understood. Therefore, aims of the present thesis work are to investigate platelet angiogenic activities, and to study the influence and mechanisms of platelet-regulated EPC angiogenic properties.

We have elucidated that platelets promoted EPC angiogenesis via the glycoproteins on the platelet surface, and identified that platelet tetraspanin CD151 and integrin α6β1, as well as EPC α6β1 were required for platelet-enhanced EPC angiogenesis. Moreover, it has been shown that platelets exerted the enhancement via Src-PI3K signalling pathway of EPCs.

Platelets contain both pro- and anti-angiogenic factors, which are stored in separate α-granules and distinctly release upon different stimulation. In paper II, we characterized that pro-angiogenic and anti-angiogenic regulators were mostly stored in separate α-granules. Furthermore, we found that protease-activated receptor (PAR) 1, adenosine diphosphate (ADP), and GPVI stimulation induced platelet secretion of pro-angiogenic regulators, whereas PAR4 stimulation selectively induced platelet secretion of anti-angiogenic regulators.

In paper III, we determined if PAR1-stimulated platelet releasate (PAR1-PR) and PAR4-PR differently regulate angiogenic properties of EPCs. To our surprise, both PAR1-PR and PAR4-PR enhanced EPC migration and tube formation. PAR1-PR enhanced vasculogenesis more potently than PAR4-PR, and the enhancements required a cooperation of multiple platelet-derived angiogenic regulators.

Platelets retain mRNAs from the megakaryocytes, and use these mRNAs as templates for de novo protein synthesis upon stimulation. In paper IV, we observed that thrombin stimulation induced SDF-1α mRNA maturation, which led to de novo synthesis of SDF-1α after activation. The data suggest that platelets may enhance angiogenesis by de novo synthesis of angiogenic regulators after activation.

Together, the thesis work demonstrates that platelets enhance EPC angiogenic properties via both secreted angiogenic regulactors and surface receptors, and that platelets may regulate angiogenesis via de novo synthesis of angiogenic regulators after activation.

LIST OF SCIENTIFIC PAPERS

I. **Huang Z**, Patarroyo M, Miao X, Nilsson G, Pernow J, Li N. Platelet membrane glycoprotein CD151 promotes endothelial progenitor cell angiogenesis. *Submitted manuscript.*

II. Chatterjee M, **Huang Z**, Zhang W, Jiang L, Hultenby K, Zhu L, Hu H, Nilsson GP, Li N. Distinct platelet packaging, release, and surface expression of proangiogenic and antiangiogenic factors on different platelet stimuli. *Blood. 2011 Apr 7;117(14):3907-11.*

III. **Huang Z**, Miao X, Luan Y, Zhu L, Kong F, Lu Q, Pernow J, Nilsson G, Li N. PAR1-stimulated platelet releasate promotes angiogenic activities of endothelial progenitor cells more potently than PAR4-stimulated platelet releasate. *J Thromb Haemost. 2015 Mar;13(3):465-76.*

IV. **Huang Z**, Rahman MF, Jiang L, Xie H, Hu H, Lui WO, Li N. Thrombin induces de novo protein synthesis of stromal cell-derived factor-1α but not angiostatin in human platelets. *J Thromb Haemost. 2012 Oct;10(10):2202-5.*

OTHER PUBLICATIONS NOT INCLUDED IN THESIS

1. Zhu L, **Huang Z**, Stålesen R, Hansson GK, Li N. Platelets provoke distinct dynamics of immune responses of different CD4+ T cell subsets via selective regulations of cell proliferation. *J Thromb Haemost. 2014 Jul;12(7):1156-65.*

2. **Huang Z**, Liu P, Zhu L, Li N, Hu H. $P2X_1$-initiated p38 signalling enhances thromboxane A2-induced platelet secretion and aggregation. *Thromb Haemost. 2014 Jul 3;112(1):142-50.*

CONTENTS

LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine/serotonin
ADP	Adenosine diphosphate
ALK1	activin receptor-like kinase-1
AMI	acute myocardial infarction
ATP	Adenosine triphosphate
BMC/BMMNC	bone marrow-derived mononuclear cells
BMP	bone morphogenetic protein
BOEC	blood outgrowth endothelial cell
BSA	bovine serum albumin
CAC	circulating angiogenic cell
CAD	coronary artery disease
CCR	CC chemokine receptor
CCK	Cell counting kit
CFU-EC	endothelial cell colony-forming unit
CLEC-2	C-type lectin-like receptor 2
CPC	circulating progenitor cell
CRP	collagen related peptide
CXCR	CXC chemokine receptor
DMEM	dulbecco's modified eagle medium
EC	endothelial cell
ECFC	endothelial colony forming cell
ECGF	endothelial cell growth factor
ECM	extracellular matrix
EPC	endothelial progenitor cell
EPDC	endothelial progenitor-derived cell
ERK	extracellular signal-regulated kinase
FGF	fibroblast growth factor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
G-CSF	granulocyte-colony stimulating factor
GP	glycoprotein
HE	hematoxylin and eosin
HPF	high-power microscope field
HSC	haematopoietic stem cell
IGF-1	insulin-like growth factor 1
IL-1	interleukin-1
MI	myocardial infarction
MMP	matrix metalloproteinases
OCS	open canalicular system
OEC	outgrowth endothelial cell
PlGF	placental growth factor
PAF	platelet-activating factor
PAI-1	plasminogen activator inhibitor-1
PAR	Protease-activated receptor
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PETA-3	platelet endothelial tetraspan antigen 3

PF4	platelet factor 4
PGI_2	Prostacyclin
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
PLG	plasminogen
PMP	platelet microparticle
PR	platelet releasate
PRP	platelet-rich plasma
PS	phosphatidylserine
S1P	sphingosine-1-phosphate
SDF-1	stromal cell-derived factor 1
SMC	smooth muscle cell
TF	tissue factor
TGFβ	transforming growth factor β
TIMP	tissue inhibitor of metalloproteinase
TP	thromboxane receptor
TSP-1	Thrombospondin 1
TXA_2	thromboxane A_2
VEGF	vascular endothelial growth factor
vWF	von Willebrand factor

1 INTRODUCTION

Angiogenesis, the sprouting of new capillaries from pre-existing blood vessels, is closely involved in many physiologic and pathologic processes [1]. The formation and persistence of new blood vessels is regulated by a complex control system. The basic steps in vessel formation include endothelial activation, proliferation, and migration, which are followed by the development of a vascular cord, lumen formation, stabilization, and finally vessel maturation [2, 3]. Identification of endothelial progenitor cells (EPCs) in the peripheral blood has highlighted an alternative mechanism of vessel formation based on the recruitment of bone marrow-derived EPCs [4-6]. Accumulating evidence in the past decade indicates that EPCs play important roles in vascular regeneration and endothelium reparation [6-8].

Platelets are essential for haemostasis [9]. At the sites of blood vessel injury, platelets are activated to induce blood coagulation and form aggregates to prevent haemorrhage and thereby protects us from fatal bleeding [10]. Besides their well-known function in haemostasis, platelets have been shown to contribute to nonhaemostatic processes, such as wound healing, immunity, angiogenesis, atherogenesis, and tumor metastasis [11-13]. More and more evidence shows that platelets play an important role in angiogenesis, and EPCs have given an alternative role in angiogenesis. Hence, the present thesis is focused on platelet-dependent angiogenic activities and how platelets regulate the EPC functions in both in vitro and in vivo experimental settings.

1.1 PLATELET PHYSIOLOGY

Platelets are the smallest blood cells, and are discoid anucleated cells (approximately 2-5 μm in diameter) shed from megakaryocytes in the bone marrow. Platelets circulate in large numbers ($200–300\times10^9$/L) under physiological conditions, and normally remain in the circulation for approximately 7 to 10 days. Platelets are in a quiescent state in circulation, and become activated by exposure to extracellular matrix (ECM). They adhere to the damaged vessel wall, build up platelet aggregates, and form platelet thrombus. These processes restore integrity of the vessel wall and stop bleeding, but may also lead to occlusive thrombus formation under pathophysiological conditions [14].

Although platelets lack nuclei, they are highly organized cells containing all other organelles, such as granules, mitochondria, and the cytoskeletal components microtubules and actin filaments. Platelets contain three different types of granules; dense granules (δ granules), lysosomes, α-granules [15]. The α-granule is the most abundant, comprising 10% of platelet volume with

approximately 50–80 α-granules per platelet [16]. The α-granules contain high molecular weight proteins and peptides, such as von Willebrand factor (vWF), fibrinogen, platelet factor 4 (PF4), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), matrix metalloproteinases (MMPs), thrombospondin 1 (TSP-1) and plasminogen activator inhibitor 1 (PAI-1) [13, 17]. The dense granules are smaller and fewer than the α-granules, and have high morphological variability [18]. Dense granules contain small molecules, such as calcium and adenine nucleotides, including adenosine diphosphate (ADP) and adenosine triphosphate (ATP). Human platelets also contain a few lysosomes. The organelles show acid phosphatase reaction product, and are spherical in form and slightly smaller than α granules. Platelets have two specialized tubular systems. The open canalicular system (OCS) is a reservoir of platelet membrane, and provides a transportation highway for the release of platelet granule contents. The dense tubular system is a calcium storage pool, and is the site for thromboxane A_2 (TXA_2) and prostaglandin syntheses [19].

Platelet receptors expressed on the cell surface determine the reactivity of platelets with a wide range of agonists and adhesive proteins. The integrins are a major class of adhesion and signalling molecules that are present on most cell types as well as on platelets. Platelets have six different integrins: α2β1, α5β1, α6β1, αLβ2, αIIbβ3, and αvβ3. Among them, αIIbβ3 is the most abundant and platelet-specific, which is the principle receptor for fibrinogen [20, 21].

Another platelet-specific receptor is glycoprotein (GP) Ib/IX/V complex, which is the primary receptor for vWF. GP Ib/IX/V complex can also bind to a number of other ligands, such as thrombin, P-selectin, factor XI (FXI), FXII, high molecular weight kininogen, as well as some bacteria membrane component [22].

The seven-transmembrane receptor family is the major agonist receptor family on platelets. For example, protease-activated receptor-1 (PAR1) and PAR4 are the receptors for thrombin, $P2Y_{12}$ and $P2Y_1$ are the receptors for ADP, and thromboxane receptor (TP) α/β is the receptor for TXA_2 [23, 24].

Immunoglobulin superfamily receptors on human platelets include GPVI and FcγRIIa. GPVI, in addition to α2β1, cooperates with FcγRIIa and serves as the signalling receptor for collagen on platelets [25]. FcγRIIa has also a role in immunological defense against bacteria, viruses, and parasites. The significance of FcγRIIa also lies in the problems caused by a variety of autoimmune and alloimmune disorders involving antigen-antibody clusters that cause platelet activation by clustering FcγRIIa [26].

The main C-type lectin receptors on platelets include P-selectin (CD62P) and C-type lectin-like receptor-2 (CLEC-2). The main function of P-selectin involves multiple, transient weak interactions with carbohydrate ligands expressed on the cells, thereby allowing the development of stronger, more stable binding via other ligands and receptors [27]. CLEC-2 has an important physiological role in vascular/lymphatic system differentiation [28].

Tetraspanins are a group of membrane proteins that, as the name implies, contain four membrane-spanning domains. They are thought to have important functions in signal transduction across the cell membrane in complexes with other membrane receptors by playing a critical role in selection of components of lipid rafts. Platelets content several members of this group of molecules, but the roles of them are still poorly understood. Platelets express at least 4 tetranspanins on the surface, CD9 (Tspan 29), CD63 (Tspan 30), CD151 (Tspan 24, platelet and endothelial cell tetraspan antigen 3 [PETA-3]) and Tspan32 [29, 30].

Besides these receptors, platelets express other type receptors on the surface, such as thrombopoietin receptor (c-mpl, CD110), PDGF receptor, CD36 (GPIV, GPIIIb), CD40 Ligand (CD40L, CD154), and so on.

1.1.1 Platelet activation

Platelets are sensitive cells, and can be activated by a number of stimuli. Table 1 lists a panel of common agonists and their corresponding receptors on platelets. The potencies of platelet agonists differ considerably. Thrombin, the most potent physiological agonist of platelets, activates human platelets via the cleavage of PAR1 and PAR4 to induce full platelet activation, including platelet shape change, adhesion and aggregation, secretion, as well as vesiculation (microparticle generation). Some platelet agonists, such as ATP and epinephrine, are so-called weak agonists, which only induce platelet shape change and/or reversible platelet aggregation.

Table 1. Main agonists and their receptors of human platelets

Agonists	Receptors	Main functions
thrombin	PAR1, PAR4	Induces full platelet activation [31, 32]
TXA_2	TP α/β	Acts as a positive feedback lipid mediator following platelet activation [33, 34]
ADP	$P2Y_1$, $P2Y_{12}$	Acts as a positive feedback mediator following platelet activation [35-37]; induce reversible platelet aggregation [38]
serotonin (5-HT)	$5HT_{2A}$	Acts as a priming agonist and a positive feedback mediator following platelet activation [39, 40]
fibrinogen/fibrin	αIIbβ3	Initiates platelet adhesion at low shear [41-43]; act as a bridge in platelet-platelet aggregation [44, 45]
vWF	GPIb-IX-V	Decrease platelet velocity and initial tethering at high shear rate [41, 42, 46-48]; act as a bridge in platelet-platelet aggregation [49]
collagen	GPVI, $\alpha_2\beta_1$	For platelet firm adhesion and platelet activation at the sites of damaged vessel [41-43]
ATP	$P2X_1$	Amplifies platelet activation induced by collagen, thrombin, and TXA_2 [50-53]
podoplanin	CLEC-2	Induces platelet activation, and help the separation of the vascular/lymphatic system [54]

Upon a blood vessel injury, ECM proteins, such as vWF and collagen, are exposed to platelets and interact with their primary platelet receptors, GPIb-IX-V and GPVI, respectively, to regulate initial platelet activation and adhesion [47, 55]. VWF is a large, multimeric GP synthesized by endothelial cells (ECs) and megakaryocytes. It is abundant in plasma but does not interact with circulating platelets, because the GPIbα binding site on vWF A1 is cryptic. After immobilization onto subendothelial surfaces through the binding to ECM components, vWF changes to an active conformation upon exposure to hemodynamic forces, and facilitates interaction with GPIbα [56]. VWF binding on platelets is reversible, but yet crucial to the sequential steps of platelet adhesion

and activation in decreasing platelet velocity, facilitating rolling, and stabilizing platelet adhesion through interaction with collagen [45].

Platelets tether on collagen via GPVI. The ligation further increases affinity and clustering of GPVI, and induces inside-out signalling leading to the activation of α2β1, another collagen receptor of platelets, and αIIbβ3. These complex adhesion molecule cross-talks coordinate their adhesion affinity, and enhance stability of platelet adhesion [57, 58].

αIIbβ3 (GPIIb/IIIa) is the most abundant membrane protein on platelets, and mediates aggregation and firm adhesion of platelets. αIIbβ3 can bind to many ligands, including fibrinogen, fibronection, vWF, TSP-1, and CD40L [59]. αIIbβ3 binds to its primary ligand fibrinogen that bridges adjacent platelets, leading to platelet aggregation and eventually thrombus formation. Moreover, αIIbβ3-ligand binding induces outside-in signalling, which leads to and/or enhances further platelet activation, such as cytoskeletal change, platelet spreading, and granule secretion [60, 61].

Except adhesion and aggregation, activated platelets undergo granule secretion. Platelet secretion releases granule contents through granule fusion with cell membrane or via OCSs. Platelets release a wide range of substances. Table 2 lists a panel of angiogenic regulating factors released from platelet α granules. Platelet dense granules release low molecular weight substences, such as ADP, ATP and serotonin, which can, in turn, stimulate platelets to amplify their activation. Platelet released substances exert various actions on platelet themselves and/or on adjacent/distant platelets and other types of cells, to amplify platelet activation and to regulate the functions of other cells. The interesting thing is that, platelets may store pro- and anti-angiogenic regulators in separate α-granules, and release differentially upon different stimuli according to recent studies [62, 63]. However, there is also evidence showing that platelet angiogenic regulators may be randomly packed into platelet α-granules but released with a distinct protein cluster [64], and that the distinct release may depend on activation intensity and secretion kinetics of platelets [65]. Moreover, distinct platelet angiogenic regulator releases upon different stimuli have been reported to exert counteracting effects on angiogenesis [66]. It is necessary to investigate if the distinct packaging and releasing of platelet pro- and anti-angiogenic factors is a general phenomenon.

Table 2. Angiogenic regulators that are released from platelets

	Regulators	Main functions
pro-angiogenic regulators	VEGF	Promotes EC proliferation, tube formation in vitro, and angiogenesis in vivo [66-70]
	PDGF	Promotes EC migration and angiogenesis in rat aortic ring [69-71]
	bFGF	Promotes EC proliferation, tube formation, and angiogenesis in rat aortic ring [68-70]
	IGF-1	Promotes angiogenesis in rat aortic ring [67, 72]
	SDF-1α	Promotes differentiation of cultured $CD34^+$ cells to EPCs and induces recruitment of $CD34^+$ progenitor cells in vivo [73-77]
	MMPs	Promote EC tube formation [78, 79]
	S1P	Promotes EC proliferation, tube formation, and angiogenesis in vivo [79-82]
	ECGF	Promotes EC migration and angiogenesis in vivo [83-88]
anti-angiogenic regulators	TSP-1	Inhibits EC proliferation and migration, as well as stimulates EC apoptosis [89, 90]
	PF4	Inhibits EC proliferation, migration, and angiogenesis in vivo [91-94]
	Angiostatin	Inhibits EC tube formation and induces EC apoptosis [95, 96]
	Endostatin	Inhibits EC proliferation, migration and tube formation, as well as induces EC apoptosis [97-99]
	TIMPs	Inhibit EC proliferation and tube formation [100-102]

Platelet activation also triggers the metabolisms of platelet membrane phospholipids, leading to the synthesis and release of TXA_2 and platelet-activating factor (PAF). Platelet activation is also an indispensable component of coagulation cascade. Most remarkedly, membrane phosphatidylserine (PS) exposure of activated platelets provides a docking site for FXa and FVa

to form prothrombinase complex, which converts prothrombin to thrombin. Thrombin augments platelet aggregation via PAR1 and PAR4 on human platelets [103].

Platelets lack nuclear DNA, however, they retain some mRNAs from the megakaryocytes, and they can use residual mRNAs as templates for de novo protein synthesis, such as Bcl-3, TSP-1, and PAI-1, upon activation [104]. Important insights have emerged recently on the regulatory controls of gene expression in human platelets, as the maturation of interleukin-1β (IL-1 β) and tissue factor (TF) mRNAs has been reported to occur through mRNA splicing after activation [105-108].

Furthermore, besides classical platelet functions in thrombosis and haemostasis, an accumulating body of evidence indicates that platelets are not a simple thrombocyte, but are versatile cells closely involving in other physiological and pathophysiological processes, such as tissue regeneration and angiogenesis. Indeed, platelets have been recognized as a major reservoir of angiogenic regulators [13, 17].

1.2 ENDOTHELIAL CELL AND ENDOTHELIAL PROGENITOR CELL

ECs are the fundamental elements of the vascular system. They form a single cellular layer that lines the interior surface of blood vessels and lymphatic vessels, and serves as a dynamic border between circulating blood and the surrounding tissues, and maintains vascular homeostasis [109]. On mechanical disruption of this monolayer, the ECM is exposed and gets into contact with the blood, which induces platelet adhesion and thrombus formation. The latter facilitates leukocyte recruitment and inflammatory responses at the injured sites, and prompt atherosclerosis [110]. Indeed, EC dysfunction is an initial step in the atherosclerotic process and implicate in the cardiovascular diseases, including hypertension, coronary artery disease (CAD), chronic heart failure, peripheral artery disease, diabetes, and thrombosis [111, 112]. Thus, integrity maintenance and regeneration of the vascular endothelium is of especial importance.

EPCs, which are circulating in peripheral blood, share many similar properties with ECs. Importantly, they may provide a circulating pool of cells that can form a cellular patch at the site of dead/injured ECs. They have a potential to proliferate and to differentiate into mature ECs, and improve vascular regeneration and endothelial reparation [6, 113]. EPCs have also been studied as biomarkers for cardiovascular disease, and proposed as a potent cell-based therapy based on their capacity to stimulate vascular repair under physiological and pathological conditions. Increased recruitment and differentiation of EPCs to the sites of vessel injury can

promote vascular regeneration and endothelial integrity [7, 8, 113]. In contrast, a reduction in circulating EPC number is a surrogate marker for vascular dysfunction, cumulative cardiovascular risks, and poor cardiovascular outcomes in patients with CAD [114-117].

1.2.1 Different types of EPCs and their markers

EPCs were first isolated using magnetic micro beads by Asahara [4]. Since then, distinctly different cell populations have been isolated and called EPCs. These cell populations have subsequently been shown to improve vascular function through two mechanisms (i) actual incorporation into injured endothelium with formation of a blood vessel and/or (ii) local secretion of pro-angiogenic factors with a paracrine effect on the cells actually forming the vessel. However, no specific marker can prospectively identify an EPC at present, and thus, the origin of the cell cannot be clearly defined. Three different populations of putative human EPCs, with a variety of names in the literature, have been defined using for cell culture and cell sorting protocols. A summary of different cell populations is given in Table 3.

Table 3 Subtypes of Endothelial Progenitor Cells and Their phenotypes

Circulating angiogenic cells (CACs) [7, 118, 119]	Early outgrowth endothelial progenitor cells (CFU-ECs, early EPCs and CFU-Hill) [4, 6, 115, 120, 121]	Late outgrowth endothelial progenitor cells (BOECs, ECFCs, EPDCs, late EPCs, and OECs) [5, 6, 113, 120, 122, 123]
No colony formation in culture	Colonies appear in 4-9 days in culture	Colonies appear around 7 day (umbilical blood) and 14 day (peripheral blood)
Low proliferative potential	Low proliferative potential	High proliferative potential
Do not form vascular tubes in vitro on Matrigel	Do not form vascular tubes but incorporate in EC-formed capillaries in vitro on Matrigel	Form vascular tubes in vitro on Matrigel
Do not form vessels in vivo	Do not form vessels in vivo	Form vessels in vivo
Home to ischemic sites in vivo	Home to ischemic sites in vivo	Home to ischemic sites in vivo
Augment angiogenesis by paracrine	Augment angiogenesis by paracrine	Low cytokine release
$CD34^{+/-}$, $CD133^{+}$, $VEGFR2^{+}$, $CD45^{+}$, $CD14^{+}$, $CD115^{+}$, CD31+, $ALDH^{bright}$, acLDL uptake, UEA-1 lectin binding	$CD34^{+/-}$, $CD133^{+}$, $VEGFR2^{+}$, $CD45^{+/-}$, $CD14^{+/-}$, $CD115^{+}$, $CD31^{+}$, $ALDH^{bright}$, acLDL uptake, UEA-1 lectin binding	$CD34^{+}$, $CD133^{-}$, $VEGFR2^{+}$, $CD45^{-}$, $CD14^{-}$, $CD115^{-}$, CD31+, $ALDH^{bright}$, acLDL uptake, UEA-1 lectin binding, $CD105^{+}$, $CD146^{+}$, $CD144^{+}$

1.2.2 EPCs for cardiovascular regeneration

Myocardial infarction (MI) is one of the most frequent causes of morbidity and mortality worldwide. Modern medicine has improved prevention of MI and the prognosis following a MI, but the mortality rates remain high [124]. Neovascularization is an important adaptation to rescue the damaged tissue from severe ischemia. Accumulated evidence has elucidated that EPCs provide a postnatal vasculogenetic mechanism for neovascularization and vascular remodelling [6,

7]. EPCs have a diverse of physiological functions and participate in the recovery processes of myocardial ischemia and infarction [8, 125, 126], limb ischemia [7, 120], wound healing [127, 128], atherosclerosis [129], and endogenous endothelial repair [130]. Since then, the investigators have begun to evaluate the potential therapeutic impacts of EPCs in ischemic diseases.

1.2.2.1 EPC Transplantation for Peripheral Ischemia

The transplantation of EPCs significantly improved blood flow recovery and capillary density in several animal models of hind limb ischemia. It has been shown that intravenous infusion of ex vivo expanded human EPCs (hEPCs) after ischemia improves neovascularization in animal models, and that histological examinations confirmed hEPC incorporation and differentiation into ECs [7, 131-134]. In contrast, infusion of mature ECs did not affect neovascularization after hind limb ischemia [7, 132]. Furthermore, EPC transplantation induces blood flow recovery in the ischemic hind limbs of both diabetic mice and rats, suggesting that EPC-mediated neovascularization can still occur under disease conditions and thus be applied as a therapeutic treatment in the patients who would benefit most [128].

1.2.2.2 EPC Transplantation for Ischemic Myocardium

Just as EPC transplantation restored blood flow to ischemic hind limbs, it also induced neovascularization after MI. Transplantation of the ex vivo expanded hEPCs to MI model had a favourable impact on the preservation of left ventricular function and reduced infraction size [8, 126]. Similar findings were documented by Kocher et al. [135] by the transplantation of G-CSF–mobilized $CD34^+$ human cells, which contain both haematopoietic stem cells (HSCs) and EPCs. The transplantation was shown to improve myocardial function, protect cardiomyocytes against apoptosis, and induce myocardial remodelling [135]. In addition, it has been demonstrated that EPC therapy improves regional systolic function accompanied by cardiac hypertrophy in porcine acute myocardial infarction (AMI) models [136].

1.2.2.3 EPC Transplantation in Humans

Initial results from clinical trials assessing the safety and feasibility of autologous progenitor cell transplantation are rather promising. A pilot clinical trial showed that transplantation of adult progenitor cells by intracoronary infusion was feasible and safe in patients with AMI, and may have beneficial effects for postinfarction remodelling processes. Since then, more and more clinical trials have been showing that intracoronary infusion of progenitor cells (either BMCs/ BMMNCs [bone marrow-derived mononuclear cells] or CPCs [circulating progenitor cells]) are

safe and feasible in patients after AMI, and have disclosed a potency-effect relationship between cell therapy and long-term outcome in patients with AMI [137-145].

Although the preclinical and clinical studies generally give a strong support to the therapeutic potential of EPCs in the treatment of cardiovascular diseases, the clinical application of EPCs is limited by several factors. At first, the relative shortage of circulating EPCs makes it difficult to expand sufficient number of cells for therapeutic application without inducing the risk of culture-induced cellular senescence and functional impairment, such as by freezing and thawing [113, 146]. Furthermore, the availability of EPCs is sensitive to some pathologic state, such as aging and diabetes, which are commonly accompanied by cardiovascular diseases [147-150]. This severely restricts the ability of autologous EPCs to treat patients with cardiovascular diseases. Finally, for a successful therapeutic EPC-based approach, it is essential to get optimal quality/quantity of EPCs through various means, such as ameliorating EPC purification and expansion methods, improving the administration and cellular application techniques, and recovering the disease-based dysfunction and/or senescence of patient-derived EPCs.

1.2.3 Angiogenesis

The importance of the circulatory system is evidenced by its early emergence in development. In vertebrates, the circulatory system is the first functional organ system to arise and is critical in providing adequate oxygen and nutrient delivery to rapidly developing tissues, above what can be provided by diffusion alone. The vasculature is formed through three main cellular processes: vasculogenesis, angiogenesis and arteriogenesis. Vasculogenesis, the de novo formation of blood vessels, gives rise to the first blood vessels, establishing a primary vascular plexus. Angiogenesis, the growth of blood vessels from pre-existing blood vessels, allows for dramatic expansion of the vascular plexus, while arteriogenesis involves an increase in arterial vessel diameter in response to increased blood flow or shear stress. Through these three mechanisms a circulatory system is formed and remodelled into a complex vessel system that mediates a wide range of vital physiological processes including tissue oxygenation, nutrient delivery, waste removal, immune response, temperature regulation, and the maintenance of blood pressure.

Angiogenesis is defined as the formation of new vessels by the sprouting of ECs of pre-existing vessels or intussusceptive angiogenesis, the translumenal insertion of tissue pillars within existing capillaries to form new vessels. In healthy adults, blood vessels are usually quiescent until activated during processes such as wound healing, the female reproductive cycle and

pregnancy. Similar to healthy tissues, the growth and progression of cancer is highly dependent on angiogenesis. Therefore, anti-angiogenesis can be an effective therapy against tumor growth if deprivation of tumor cells from oxygen and nutrients is effectively achieved. It has stirred immense interest in the research community, and many therapeutic drugs have been developed to target angiogenesis dependent diseases including cancer, ophthalmic diseases, arthritis, psoriasis, obesity and obesity-related metabolic diseases.

Much research into the mechanisms of angiogenesis followed after Judah Folkman proposed the inhibition of angiogenesis as a means of tumor treatment [151]. Based on an array of studies, we now understand that sprouting angiogenesis is a coordinated series of events centered on ECs [152, 153]. Vascular sprouts are led by specialized endothelial “tip cells” that are responsive to angiogenic stimuli [154] and connected to endothelial stalk cells that function in tube formation. The progression of angiogenesis is initiated by local destruction of the basement membrane of a vessel and the dissociation of pericytes from the capillary, followed by migration of tip cells toward an angiogenic stimulus. Proliferation and alignment of ECs follows as an EC tube formation, establishing a lumen. Pericyte and/or smooth muscle cell association and basement membrane deposition mediate vessel stabilization.

1.2.4 Regulation of angiogenesis

Angiogenesis is highly governed by the balance of angiogenic stimulators and inhibitors. Therefore, pathological conditions, including cancer, diabetes and macular degeneration, arise when this balance is tipped.

1.2.4.1 Pro-angiogenic factors

1.2.4.1.1 Vascular endothelial growth factor (VEGF)

VEGFs and theirs receptors are the best-characterized signalling pathway involved in regulations of both vasculogenesis and angiogenesis. The VEGF protein family consists of VEGF-A (also known as VEGF), VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF) [155]. Of these, VEGF-A, originally identified as vascular permeability factor [156] has been the most widely studied VEGF family member and has been implicated in both vasculogenesis and angiogenesis. Loss of a single VEGF-A allele results in embryonic lethality. VEGF-B has been shown to play a central role in cardiac development [157, 158]. VEGF-C and VEGF-D promote lymphatic vessel development [159-161] and may also contribute to angiogenesis [162, 163]. PlGF, originally identified in the placenta, occurs at low levels in the

embryo and adult and has been primarily studied in pathological conditions where it is thought to stimulate angiogenesis in coordination with VEGF-A [164]. VEGF-A is present in platelets at a concentration of about 0.74 ± 0.37 pg/10^6 platelets. The major isoforms present in platelets are VEGF-A and VEGF-C. Both promote permeability of the vessel wall and serve as a chemoattractant for EC sprouting in the initial stage of angiogenic response [225, 226].

VEGFs act through three structurally related VEGF receptor tyrosine kinases, denoted VEGFR1 (Flt1), VEGFR2 (Flk1/KDR), and VEGFR3 (Flt4). The receptors show an overlapping but distinct expression pattern. The continuously increased sensitivity of reagents and detection methods show that there is a wider expression range of the VEGF receptors than initially anticipated. However, there is still an overall pattern of VEGFR1 expression in monocytes and macrophages, VEGFR2 in vascular ECs, and VEGFR3 is present in all endothelia during development, and in the adult it becomes restricted to the lymphatic endothelium [155, 161, 165].

VEGFR1 is widely expressed, and its kinase activity is poor and not required for EC function. An important role for VEGFR1 is in negative regulation of VEGFR2 biology, by binding VEGF, and in regulation of monocyte migration during inflammation. Moreover, the different VEGFR1 ligands have quite distinct functions, such as transport of fatty acids and regulation of pathological angiogenesis. VEGFR2 is the main VEGF receptor on ECs. VEGFR2 is essential for EC biology during development and in the adult, in physiology and pathology. More is known about VEGFR2 signalling than for the other VEGFRs. Several small-molecular-weight inhibitors of VEGFR2 kinase activity are employed clinically to block pathological angiogenesis in cancer [166]. VEGFR3 is critical regulator of lymphendothelial function. Loss-of-function VEGFR3 mutants in humans cause lymphedema. VEGFR3 signalling also positively regulates angiogenesis [165]. In particular, VEGFR3 is highly expressed in TCs and is required for EC sprouting in mice and zebrafish [165, 167]. VEGFR3 forms homodimers as well as heterodimers with VEGFR2, which are enriched in TCs and positively influence angiogenic sprouting [155, 168].

1.2.4.1.2 Fibroblast growth factor (FGF)

FGF is another pro-angiogenic growth factor, which is stored in the vascular basement membrane to serve as a reservoir supply, and is upregulated during active angiogenesis. The two most commonly studied forms are FGF-2 or basic FGF (bFGF) and FGF-1 or acidic FGF (aFGF), which bind most commonly to the receptor tyrosine kinases FGFR1 or FGFR2. FGF

plays important roles in a wide variety of physiological and pathological processes, including angiogenesis, vasculogenesis, wound healing, tumorigenesis, and embryonic development [169-174]. In vitro, FGF increases EC migration, and promotes capillary morphogenesis on collagen gels [175, 176]. In addition, bFGF mediates proteolysis of matrix components (via up-regulation of urokinase receptors), and induces the synthesis of collagen, fibronectin and proteoglycans by ECs, and thereby excerts its effects on ECM remodelling during angiogenesis [175, 177].

1.2.4.1.3 Platelet-derived growth factor (PDGF)

PDGF is a potent mitogen for cells of mesenchymal origin, including smooth muscle cells (SMCs) and glial cells. Although α-granules of platelets are a major storage site for PDGF, recent studies have shown that PDGF can be synthesized by a number of different cell types, such as ECs and activated macrophages [178]. PDGF is present in platelets at a concentration of about 23 ± 6 pg/10^6 platelets. In both mouse and human, the PDGF signalling network consists of four ligands, PDGFA-D, and two receptors, PDGFRα and PDGFRβ. Most PDGFs function as secreted, disulfide-bonded homodimers, but only PDGFA and B can form functional heterodimers [179].

The PDGFRs are expressed on capillary ECs, and PDGF has been shown to have an angiogenic effect [178]. The effect is, however, weaker than that of FGFs or VEGFs, and PDGF does not appear to be of importance for the initial formation of blood vessels, since no apparent vascular abnormality was observed during early embryogenesis in mice with genes for PDGF or PDGFRs inactivated [180]. In contrast, PDGF-BB/PDGFRβ signalling pathway is crucial to recruit mural cells (pericytes and SMCs) to blood vessels to maintain structural integrity [180, 181]. Mice lacking PDGFB or PDGFRβ display a profound decrease in the number of SMCs and pericytes associated with the vessels, leading to unstable, leaky vessels and irregular vascular networks [180, 182].

1.2.4.1.4 Transforming growth factor-β (TGF- β)

The TGFβ family encompasses an array of members including the TGFβs (TGFβ1, TGFβ2, and TGFβ3), bone morphogenetic proteins (BMPs), growth and differentiation factors, Activins and Nodal. TGFβ family members bind to two types of receptors, type I and type II [183]. They are secreted in an inactive latent form, which requires cleavage of the latency associated peptide domain by proteases, often under acidic conditions.

TGF-β1 has multiple effects on vascular ECs. In vivo, TGF-β1 induces angiogenesis. Half of

mice genetically deficient in TGF-β1 die at E9.5-E10.5 due to defective yolk sac vasculogenesis [184]. Ablation of TGFβRII also results in embryonic lethality at E10.5 due to defective vasculogenesis in the yolk sac and embryo [185, 186]. In humans, mutations in endoglin, a part of the TGFβ receptor complex, and Activin receptor-like kinase-1 (Alk1) result in hereditary hemorrhagic telangiectasia, a vascular disorder characterized by arteriovenous malformations, severe bleeding [187, 188]. However, in vitro TGF-β1 inhibits EC proliferation [189], migration and proteolytic activity [190], downregulates VEGFR2 expression [191], and induces EC apoptosis [192-194].

1.2.4.1.5 Angiopoietin (Ang)

Ang is a member of a family of vascular growth factors that play a role in embryonic and postnatal angiogenesis. The Ang family of growth factors is comprised of four family members: Ang1, Ang2, Ang3 and Ang4. Angs bind the second immunoglubulin motif of Tie2 whereby they activate Tie2 and, indirectly, Tie1 in Tie1/Tie2 heterodimers [195].

Ang-1-deficient mice die between E11.5 and E12.5 because the embryos are unable to form a complex vascular network and exhibit decreased vessel support by mural cells [196]. Transgenic Ang1 overexpression or systemic adenoviral delivery resulted in increased vascular branching [197-199]. In vitro experiments show the similarly results. Ang1 can induce EC proliferation, migration, tube formation and sprouting [200-204], and enhances survival from a variety of apoptotic insults [205-208].

The role of Ang2 in blood vessel regulation is quite complex. Transgenic overexpression of Ang2 leads to a phenotype essentially the same as that seen in the Ang1 knockout, suggesting that Ang2 serves as an antagonist for Ang1 [209]. In vitro, Ang2 can prevent Ang1-stimulated effects on ECs including migration and Tie2 phosphorylation. Interestingly, it has also been shown that Ang2 can activate EC Tie2 at high concentrations [210] or when ECs are plated on fibrin or collagen matrix [211, 212]. Indeed, the Ang2 knockout demonstrates that, while Ang2 is dispensable for embryonic vascular development, Ang2 is required for both the vascular regression and sprouting events involved in postnatal ocular angiogenesis [213].

The less well-studied members of the family, Ang3 and Ang4, are interspecies orthologues between mouse and human, respectively. They have different tissue distributions: Ang3 is expressed in multiple mouse tissues, whereas Ang4 is specifically present at high levels only in human lungs. Ang4 phosphorylates Tie2, whereas Ang3 not only fails to phosphorylate Tie2, but

it even inhibits Ang1-induced phosphorylation of Tie2 in human EC [214]. Subsequent studies have demonstrated that both Ang3 and 4 are agonists of Tie2 receptor signalling, with Ang3 being a specific ligand for Tie2 receptors of its own species [215]. Consistent with this notion, both Ang3 and Ang4 are able to induce angiogenesis in vivo using the mouse corneal micropocket assay [215].

1.2.4.1.6 Stromal cell-derived factor-1 (SDF-1)

SDF-1 (CXCL12) is a constitutively expressed and inducible chemokine that regulates multiple physiological processes, including embryonic development and organ homeostasis [216]. SDF-1α, which is the predominant isoform found in all organs, is produced by megakaryocytes in the bone marrow and contained in α-granules of platelets. It has been shown that platelet-derived SDF-1α acts as a chemoattractant and homing signal for $CXCR4^+$ EPCs to the sites of neovascularization in ischaemic tissues [73, 77]. Other $CXCR4^+$ proangiogenic cells are composed of immature and mature hematopoietic cells and SMC progenitors, which all have direct or indirect proangiogenic properties, are also recruited to the sites of neovascularization in ischaemic tissues. Platelet derived SDF-1α has been reported to promote differentiation of cultured $CD34^+$ cells into EPCs [76]. Recently, it has been shown that platelet-derived SDF-1α plays a critical role in lung alveoli regeneration. After pneumonectomy, platelets-derived SDF-1α stimulates the receptors CXCR4 and CXCR7 on pulmonary capillary endothelial cells to deploy the angiocrine membrane-type metalloproteinase MMP14, stimulating alveolar epithelial cell expansion and neoalveolarization [75].

1.2.4.2 Anti-angiogenic factors

Angiogenic inhibitors can be categorized into endogenous and exogenous. Endogenous inhibitors include TSP, angiostatin, and endostatin, which exert their effect through inhibition of EC survival, proliferation and migration.

1.2.4.2.1 Thrombospondin (TSP)

TSP is a family of multifunctional proteins. The family consists of thrombospondins 1-5. TSP-1, initially isolated from human platelets [217], became the first endogenous inhibitor of angiogenesis to be identified [218]. It is found in concentrations of 31 ± 12 ng/10^6 platelets. In the tumor environment, TSP-1 and TSP-2 serves as potent endogenous inhibitors of angiogenesis by activating TGFβ, thus suppressing tumor angiogenesis. TSP-1 antagonizes VEGF in several important ways, via inhibition of VEGF release from the extracellular matrix,

direct interaction, and inhibition of VEGF signal transduction [89, 90, 218].

1.2.4.2.2 Endostatin

Endostatin is a fragment of collagen XVIII that is present in the vessel walls and basement membranes of the vasculature, and plays an important role in EC adhesion and cytoskeletal organization. The concentration of endostaton in platelets is 5.6 ± 3.0 pg/10^6 platelets. Endostatin induces EC apoptosis and blocks VEGF-induced migration in ECs, inhibits tumor growth, and impairs blood vessel maturation in wound healing. It is thought to interfere with the proangiogenic actions of growth factors, such as bFGF and VEGF [97, 98, 219]. However, clinical trials showed that endostatin did not result in a significant tumor regression in patients with advanced neuroendocrine tumors [220, 221].

1.2.4.2.3 Angiostatin

Angiostatin is a fragment of plasminogen (PLG), which is a cleavage product of several enzymes, such as urokinase and tissue-type plasminogen activator. It has both potent antiangiogenic activities and anti-proliferative activities toward ECs and cancer cells [222]. Recent evidence supports dual antitumor mechanisms for PLG derivatives, one affecting angiogenesis and another targeting tumor cells directly [223]. Kringle 5 (K5), like angiostatin, is a by-product of the proteolytic cleavage of PLG. A recent study demonstrated that K5 functions as a competitive antagonist of hepatocyte growth factor [224].

1.3 THE ROLE OF PLATELET IN ANGIOGENESIS

The first scientific evidence suggesting that platelets affect vascular endothelium in a way that constitutes a basis for new vessel development was reported in the late 1960s [225]. It was demonstrated that perfusion of organs with platelet-depleted plasma caused instability of the endothelial layer, parenchymal degeneration, and haemorrhages. The addition of platelets markedly reduced this injurious effect. In subsequent animal experiments, thrombocytopenia was associated with a higher vascular permeability to blood cells and plasma constituents that appeared to result from large gaps between ECs. A number of studies suggested that platelets promote EC proliferation. As a result of this early research, vascular biologists in the 1960s and 1970s considered platelet interactions with the vascular wall more trophic or nutritious than related to new vessel development. Nevertheless, this research created the foundation for the development of the current concepts of platelet involvement in the angiogenic response. Besides

the mediators release from α- granules, platelets influence angiogenesis through other two distinct mechanisms, the release of microparticles and direct interaction via ligand-receptor interactions.

1.3.1 Platelet-derived microparticles

Platelet-derived microparticles (PMPs), the small plasma membrane vesicles (0.1-1 μm) shed from platelets upon their activation, constitute approximately 70–90% of microparticles in the blood stream [226] and are suggested to be involved in thrombosis, inflammation and angiogenesis [227, 228]. Microparticles facilitates communication between neighbouring cells via several different mechanisms; by affecting direct cell-cell contacts, by their function as transport vesicles carrying and transferring proteins and mRNA between cells, and by direct regulation of cell signalling.

The first suggestion of a PMP-angiogenesis link stemmed from the observation that patients with gastric cancer had markedly increased plasma levels of PMPs, which showed a positive correlation with levels of proangiogenic factors such as VEGF [229]. Later, it has been shown that PMPs could promote EC proliferation, survival, migration and tube formation in vitro [228]. It was reported that the active components of PMPs eliciting these responses involved the collaborative effect of an unknown lipid component, presumably S1P and the growth factors, VEGF and bFGF. Brill et al. [70] extended these findings showing that PMPs, released by thrombin-activated platelets induced angiogenesis and improved revascularization following myocardial ischemia in vivo. Furthermore, PMPs have been shown to modulate functional features of EPCs, which are crucial for their regenerative potential. Treatment of cells with PMPs increased expression of mature EC markers on the progenitor cells and promoted both EC adhesion and paracrine activity, leading to improved endothelial regeneration. Similar to growth factors, PMPs stimulated the growth of EPCs ex vivo, presumably through regulation of intracellular signalling pathways involving ERK and phosphoinositide 3-kinase (PI3K)/Akt [228]. More recently, Laffont et at. [230] reported that PMPs were internalized by ECs and regulated expression of endogenous mRNA levels in ECs via the miRNA in PMPs. Despite PMPs representing a relatively new discipline in the field of platelet research, their growing role in a range of clinical-based studies highlights their therapeutic relevance, both as a prognostic marker for various diseases and new targets for anti-platelet therapies involving thrombotic complications and angiogenesis-related disorders.

1.3.2 Receptors-Ligand interactions

The first suggestion of platelet regulated angiogenesis through receptor-ligand interactions

stemmed from the observation that physical presence of platelets themselves but not the releasate from activated platelets is necessary for platelet-promoted EC tube formation in Matrigel [231]. Later, it has been shown that platelets promote EC proliferation through P-selectin and CD40L [232].

SDF-1α, which is found in platelet α-granules, expresses on the activated platelets and recruits of $CD34^+$ progenitor cells to arterial thrombi in vivo [73, 77]. SDF-1α also promotes differentiation of cultured CD34+ cells to EPCs [73, 76, 77].

2 AIMS OF THE STUDY

The overall aim of the thesis work is to investigate the role of platelets in angiogenesis and improve our understanding of molecular mechanisms underlying platelet angiogenic activities. Specifically, we aimed to:

- Investigate platelet-regulated angiogenic activities of EPCs (**Paper I**)
- Study the distinct packaging and release of pro- and anti-angiogenic regulators by platelets and their effects on EPC functions (**Paper II and Paper III**)
- Elucidate potential impact of platelet activation on de novo protein synthesis of platelet-derived angiogenic regulators (**Paper IV**)

3 METHODS

3.1 PLATELETS ISOLATION (PAPER I-IV)

All blood donors had antecubital veins that allowed a clean venepucture, and denied taking any medication during the 2 weeks preceding venepuncture. Blood was drawn without stasis using the siliconized vacutainers containing 1:9 (v/v) 3.8% sodium citrate.

For preparation of platelet-rich plasma (PRP), whole blood was centrifuged at 190 ×g for 20 min, and upper 2/3 of PRP were collected for further experiments.

For preparation of washed platelets, the PRP was further centrifuged at 900 ×g for 10 min in the presence of 1 μM prostacyclin (PGI_2; Sigma-Aldrich; St Louis, MO, USA), and the pellet was resuspended in Tyrode's Hepes buffer containing 1 μM PGI_2. Platelets were pelleted again and then resuspended in Tyrode's Hepes buffer. In some cases, PGI_2 was replaced by the PGI_2 analogue iloprost (Ciba Geigy), which irreversibly inhibits platelet reactivity.

For preparation of leukocyte-depleted washed platelets, PRP were isolated from venous blood as described above. Contaminating leukocytes in PRP were removed by $CD45^+$ bead selection. Washed platelets (leukocyte contamination of < 0.001%) were resuspended in serum-free Dulbecco's modified Eagle's medium (DMEM; Life Technologies; Waltham, MA, USA) at a concentration of 10^9 mL^{-1}, and treated (37 ℃, 30 min, or 16 h) with vehicle or thrombin (0.1 U mL^{-1}; Sigma-Aldrich).

3.2 PREPARATION OF PLATELET RELEASATES (PAPER I, II & III)

Washed platelets were stimulated with PAR1-activating peptide (PAR1-AP 10 μM; SFLLRNPNDKYEPF-OH from Calbiochem), PAR4-AP (100 μM; AYPGKF-NH2, from Sigma-Aldrich), ADP (Sigma-Aldrich), collagen related peptide (CRP; 1 μg/mL, from Dr R. Farndale, Cambridge, United Kingdom), or U46619 for 10 min at 37 ℃. Platelets were stimulated for 10 min at 37 ℃, the samples were centrifuged at 15000 ×g for 10 min at 4 ℃, and the supernatant was collected and stored at -80 ℃.

Total platelet releasate was prepared by three freeze/thaw cycles. After centrifugation at 13000 ×g for 10 min at 4 ℃ to deplete debris, the platelet lysate was aliquoted and stored at - 20 ℃, and was used within 4 weeks.

3.3 FLOW CYTOMETRIC ANALYSIS (PAPER II-IV)

3.3.1 Platelet surface marker analyses (PAPER II)

Aliquots of 5 µl platelets were added to 45 µl of HEPES-buffered saline containing appropriately diluted antibodies for the detection of platelet P-selectin (BD; San Diego, CA, USA), PF4 (R&D systems; Abingdon, UK), SDF-1α (R&D systems), endostatin (Hycult Biotech; Uden, The Netherlands) or VEGF (R&D systems) expression and in the presence of vehicle, PAR1-AP, ADP, CRP, U46619, or PAR4-AP. The samples were incubated for 20 min before fixation/dilution with 0.5% (v/v) formaldehyde saline. Platelet P-selectin, PF4, SDF-1α, and VEGF expression was reported as the percentages of marker-positive cells in the total platelet population.

3.3.2 Purity of washed platelets (PAPER IV)

Purity of the washed platelets was detected by flow cytometry using FITC-GPIX (BD) and PE-CD45 (Beckman-Coulter; Hialeah, FL, USA). Flow cytometric analysis of the entire cell population demonstrated that CD45-depleted platelet preparations contained less than < 0.001% CD45 positive cells.

3.3.3 Characterization of EPCs (PAPER III)

Early-passage (1-5) EPCs (5×10^4) were detached by trypsinization (0.01% trypsin/5 mM EDTA; Sigma-Aldrich) and incubated at 4 ℃ for 30 to 60 minutes with optimal concentrations of fluorescent antibodies or isotype control antibodies in 50 µl PBS with 2% FBS. The samples were then washed 2 times, and analyzed using a Beckman-Coulter FC500 flow cytometer.

3.3.4 EPC apoptosis and cell cycle assay (PAPER III)

EPCs (2.5×10^4 in 1000 µL complete medium) were cultured in a 12-well flat-bottom plate. After 24 h culture, the medium were replaced by EBM-2 SingleQuot medium without or with platelet releasates and cultured for further 18 h. For apoptosis assay, EPCs were harvested, washed with PBS, and processed for Annexin V-FITC and propidium iodide (PI) staining with an Annexin V-FITC kit (Beckman Coulter). For cell cycle analysis, the cells were harvested and resuspended in PBS at the concentration of 10^5 cells/ml. The cells were fixed with 70% cold ethanol and stored at 4 ℃ overnight, and they were then washed with cold PBS and centrifuged. The cells were resuspended in 0.25 ml PBS containing RNase (0.2 mg/ml) and incubated at 37 ℃ for 1 h. Afterwards, the cells were then labelled with PI (1 mg/ml; 1 min). Both samples of apoptosis and cell cycle analyses were performed using a FC500 flow cytometer.

3.4 IMMUNOFLUORESCENCE MICROSCOPY (PAPER II & III)

PRP (2×10^8 cells/mL) was subjected to the treatment with vehicle (resting), PAR1-AP (10 μM), PAR4-AP (100 μM), ADP (10 μM), and CRP (1 μg/mL) for 5 minutes at room temperature. Thereafter, PRP was fixed for 20 minutes in suspension by the addition of an equal volume of 4% paraformaldehyde. Fixed platelets in suspension were placed in wells of a 24-well plate, each containing a 0.01% poly-L-lysine-coated coverslip, the plate was centrifuged at 250 ×g for 5 minutes to attach the cells to the coverslip, and then permeabilized with 0.5% Triton X-100 in PBS. After blocking with 1% bovine serum albumin-PBS, the samples were incubated overnight at 4 ℃ with respective primary antibodies: mouse anti–human SDF-1α (R&D Systems) at 1:20; rabbit anti–human PF4 (Santa Cruz Biotechnologies; Santa Cruz, CA, USA) at 1:25; rabbit anti–human VEGF (Thermo Fisher Scientific; Waltham, Massachusetts, USA) at 1:500; and rabbit anti–human endostatin (abcam; Cambridge, UK) at 1:50. After washing, they were incubated with corresponding fluorescent secondary antibodies (DyLight 549 goat anti-mouse IgG at 1:50; AlexaFluor-488 goat anti–rabbit IgG at 1:100, AlexaFluor-546 goat anti–rabbit IgG at 1:500, and AlexaFluor-488 goat anti–rabbit IgG at 1:100/AlexaFluor-647 donkey anti–mouse IgG at 1:100 for double labeling) for 2 hours at room temperature. After thorough washings with PBS containing 0.3% Triton X-100 and 0.1% Tween-20, the coverslips were mounted with Prolong Gold antifade mounting medium (Life Technologies). Single immunofluorescence and SDF-1α/PF4-double immunofluorescence platelet images were acquired using a Leica confocal microscope TCS SP2 equipped with a 100× NA1.4 objective. The digital images were assembled into composite images using Adobe PhotoShop Version 10.0.1.

Four-μm-thick cryosections were first blocked with 5% goat serum (ab7481; abcam) for 30 min. The sections were then incubated with a rabbit anti-mouse CD31 polyclonal antibody (ab28364; abcam) or a nonspecific IgG antibody for 1 h at room temperature, which was followed by 1h incubation in the dark with fluorescein isothiocyanate–conjugated goat anti-rabbit secondary antibody (ZF-0311; ZSGB-Bio Co., Beijing, China). Fluorescent images were taken with a Nikon Eclipse 90i microscope.

3.5 IMMUNOGOLD-ELECTRON MICROSCOPY (PAPER II)

For preparation of cryosections, isolated human platelets were fixed with 4% paraformaldehyde in 0.1 M Na phosphate buffer, pH 7.4. After 2 hours of fixation at room temperature, the cell pellets were washed with PBS containing 0.2 M glycine to quench free aldehyde groups from the fixative. Before freezing in liquid nitrogen, cell pellets were infiltrated with 2.3 M sucrose in PBS for 15

minutes. Frozen samples were sectioned at -120 ℃, and the sections were transferred to formvar-carbon coated copper grids and floated on PBS until the immunogold labeling was carried out. An aliquot of 4 µl was added to a grid with a formvar supporting film coated with carbon for 5 min. The excess solution was soaked off with a filter paper, and the sample was stained with 0.5% uranyl acetate in water for 10 sec and air-dried. Grids were floated on drops of 1% BSA for 10 minutes to block for nonspecific labeling, transferred to 5-µl drops of primary antibody, and incubated for 30 minutes. The grids were then washed in 4 drops of PBS for a total of 15 minutes, transferred to 5-µl drops of Protein-A gold for 20 minutes, and washed in 4 drops of PBS for 15 minutes and 6 drops of double distilled water. For double labeling, after the first Protein A gold incubation, grids were washed in 4 drops of PBS for a total of 15 minutes and then transferred to a drop of 1% glutaraldehyde in PBS for 5 minutes and washed in 4 drops of PBS/0.15 M glycine. The second primary antibody was then applied, followed by PBS washing and treatment with different size protein A gold as above. In the present thesis work, SDF-1α was probed with 5-nm gold protein A, while PF4 was probed by 10-nm gold protein A. Contrasting/embedding of the labelled grids was carried out on ice in 0.3% uranyl acetate in 2% methyl cellulose for 10 minutes. Grids were picked up with metal loops, leaving a thin coat of methyl cellulose. The grids were examined with a Leo 906 transmission electron microscope (Leo GmbH) operating with an accelerating voltage of 80 kV and at 60 000x original magnification. Digital images were taken with a Morada camera (SiS Münster, Oberkochen, Germany).

3.6 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) (PAPER II)

Washed platelets (3×10^8/mL) were stimulated, and the supernatant was collected and aliquoted after centrifugation (14 000 ×g, 5 minutes, 4 ℃). The levels of VEGF, SDF-1α, PF4, and endostatin were determined using corresponding DuoSet ELISA kits (R&D Systems).

3.7 PROTEIN EXTRACTION AND WESTERN BLOT (PAPER IV)

Platelet suspensions (10^9/mL in serum-free DMEM) were incubated in the presence of vehicle or thrombin (0.1 U/mL) for 30 min or 16 h at 37^{o}C. Platelet pellets were lysed with an NP-40 lysis buffer (Life Technologies) containing a protease inhibitor cocktail (Sigma-Aldrich) and 1 mM phenylmethanesulfonyl fluoride (Sigma-Aldrich) after centrifugation at 2000 g for 5 minutes.

Platelet lysates were mixed with an equal volume of loading buffer containing 5% β-mercaptoethanol, and incubated for 5 min at 95^{o}C. Proteins were separated on 10% or 16%

Novex Tris-Glycine gels (Life Technologies), transferred to a nitrocellulose membrane, and then subjected to western blotting. SDF-1α and angiostatin were detected with the 460-SD and MAB926 antibodies (both from R&D Systems), respectively, which were subsequently probed with the horseradish peroxidase-conjugated goat anti-mouse IgG antibody sc-2031 (Santa Cruz Biotechnology). Signal detection was carried out with a Novex ECL chemiluminescence kit (Life Technologies). Glyceraldehyde- 3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology) was used as a loading control.

3.8 RNA EXTRACTION AND QUANTITATIVE REAL-TIME PCR (PAPER IV)

Platelet suspensions (10^9/mL in serum-free DMEM) were incubated in the presence of vehicle or thrombin (0.1 U/mL) for 30 min or 16 h at 37 ℃. Platelet pellets total RNA was extracted with a mirVana microRNA isolation kit (Ambion/Applied Biosystems, Austin, TX, USA) after centrifugation at 2000 g for 5 minutes.

mRNA expression levels of SDF-1α and angiostatin were quantified in unstimulated and thrombin-activated platelets with quantitative real-time RT-PCR (qRT-PCR) assays. TaqMan Gene Expression Assays for SDF-1α/CXCL12 (primer ID: Hs00171022_m1), angiostatin/PLG (Hs00264877_m1) and TaqMan Gene Expression Control 18S (ID: Hs99999901_s1) were from Applied Biosystems, and qRT-PCR was performed with a StepOnePlusReal-Time PCR system (Life Technologies). All real-time experiments were performed in triplicate. Data was normalized by the expression of 18S rRNA and expressed either as relative expression ($2^{-\Delta Ct}$).

3.9 EPC CULTURE (PAPER I & III)

Venous blood was centrifuged at 190 ×g for 20 min to obtain PRP. The remaining blood was diluted with same volume PBS, and is overlaid onto Histopaque 1077 (1:1,v/v; Sigma-Aldrich), and then centrifuge at 500 ×g for 30 minutes at room temperature to isolate peripheral blood mononuclear cells (PBMCs). Collect the PBMCs from the middle layer, and washed twice with PBS. At last, isolated PBMCs were resuspended with EGM-2 SingleQuots complete medium, which was composed of EBM-2 basal medium, 10% FBS, and the SingleQuots Kit. The PBMCs were seeded at $2–4\times10^6$ cells per well in a fibronectin-coated 24-well culture plate (Merck Millipore; Billerica, MA, USA) and cultured in an incubator at 37 ℃ with 5% CO_2. After 4 days, nonadherent cells were discarded, and fresh medium was applied. The adherent cells were continually cultured in the complete medium that was changed every 3 days until the first passage.

3.10 EPC PROLIFERATION ASSAY (PAPER III)

EPC proliferation was monitored using a Cell Counting Kit (CCK)-8 (Dojindo; Munich, Germany) assay. Briefly, subconfluent cells were detached by trypsinization that was followed by neutralization with the complete medium. After a wash, the cells were resuspended in the complete medium. EPCs were cultured in triplicates (2.5×10^3 in 100 μL complete medium per well) in a 96-well flat-bottom plate with the complete medium alone or the complete medium containing different concentrations of platelet releasates. After 18- and 48-h incubation, the CCK-8 colorimetric reagent WST-8 (10 μL per well) was added to each well and further incubated for 3 h. Then, the absorbance was measured at 450 nm using a microplate reader.

3.11 EPC MIGRATION ASSAY (PAPER III)

EPC migratory function was evaluated using a modified Boyden chamber (BD) with a polycarbonate filter with 8-μm pores placed between the upper and lower chambers. Subconfluent cells were detached by trypsinization that was followed by neutralization with the complete medium. After a wash with PBS, the cells were resuspended in serum-free EBM-2 medium. In some experiments, EPCs were preincubated with the VGFRE2 inhibitor Ki8751 (10 μM; Tocris Bioscience; Bristol, UK), the pan-MMP inhibitor GM6001 (20 μM; Tocris Bioscience), or the SDF-1α/CXCR4 inhibitor AMD3100 (10 μM; Tocris Bioscience) for 10 min at 4 ℃. Cell suspensions were placed in the upper chamber, and the lower chamber was filled with complete medium in the absence or presence of 10% platelet releasates. The EPCs were then cultured at 37 ℃ for 6 or 24 h. Cells remaining on the upper surface of the transwell membrane were wiped away gently with a cotton ball, and the cells that had migrated to the lower surface were fixed with 4% paraformaldehyde and stained with Giemsa. The magnitude of EPC migration was evaluated by counting the migrated cells in 10 random high-power microscope fields (HPF).

3.12 IN VITRO TUBE FORMATION ON MATRIGEL PLATE (PAPER I & III)

The tube formation assay was performed using the Matrigel (Corning; New York, USA) to assess angiogenetic capacity of the EPCs. In brief, EC Matrixgel solution was thawed at 4 ℃ overnight, and placed 50 μl/well in a 96-well plate at 37 ℃ for 1 h to allow the matrix solution to solidify. A total of 1×10^4 cells (100 μl) in EBM-2 SingleQuot medium were added into the Matrigel-coated wells and incubated at 37 ℃. Tubule formation was observed under an inverted light microscope (10×), and five representative fields from each well were photographed. The branching points of capillary structure were assessed using the Wimasis software (Munich,

Germany).

3.13 IN VIVO VASCULOGENESIS EXPERIMENTS (PAPER III)

The protocol of in vivo vasculogenesis using a murine model of Matrigel implantation was approved by the Institutional Animal Care and Use Committee of the Shandong University. Male C57BL/6 mice (8-12 weeks) were obtained from the Animal Experimental Center of the Shandong University.

Matrigel gel (350 μL) containing 10% of supernatant of unstimulated platelets (Rest-PR), PAR1-PR, or PAR4-PR was implanted on both flanks of a mouse via subcutaneous injection using a 25-gauge needle. Matrigel implants were removed 2 weeks after implantation. After Matrigel implants were photographed under a macroscope, one part of the implants was cryosectioned for fluorescent immunohistochemical examination. The other part was fixed in 10% neutralized formalin overnight, embedded in paraffin, and sectioned.

Hematoxylin and eosin (HE)–stained 4-μm-thick sections were examined for vasculogenesis under a Nikon Eclipse 90i microscope (Nikon Corporation, Tokyo, Japan).

4 RESULTS

4.1 PLATELETS PROMOTE ENDOTHELIAL PROGENITOR CELL ANGIOGENESIS VIA THEIR MEMBRANE RECEPTORS (PAPER I)

4.1.1 Development and characterization of human late EPCs

First we cultured EPC from peripheral blood. PBMCs were harvested and seeded onto fibronectin-coated wells. The cells were initially seen as small and round shaped cells. Small colonies of EPCs appeared after 2-3 weeks of culture. At confluence, EPCs exhibited a smooth cytoplasmic outline and showed a cobblestone appearance similar to cultured HUVECs. Flow cytometric phenotyping demonstrated that the EPCs, similar to HUVECs, expressed the endothelial markers CD31/platelet endothelial cell adhesion molecule (PECAM-1), KDR/VEGFR2, and CD146, but were negative for the leukocyte markers CD45, CD14, or CD115. However, some EPCs retained certain levels of CD34 expression, which was not detected on HUVECs (Fig. 1).

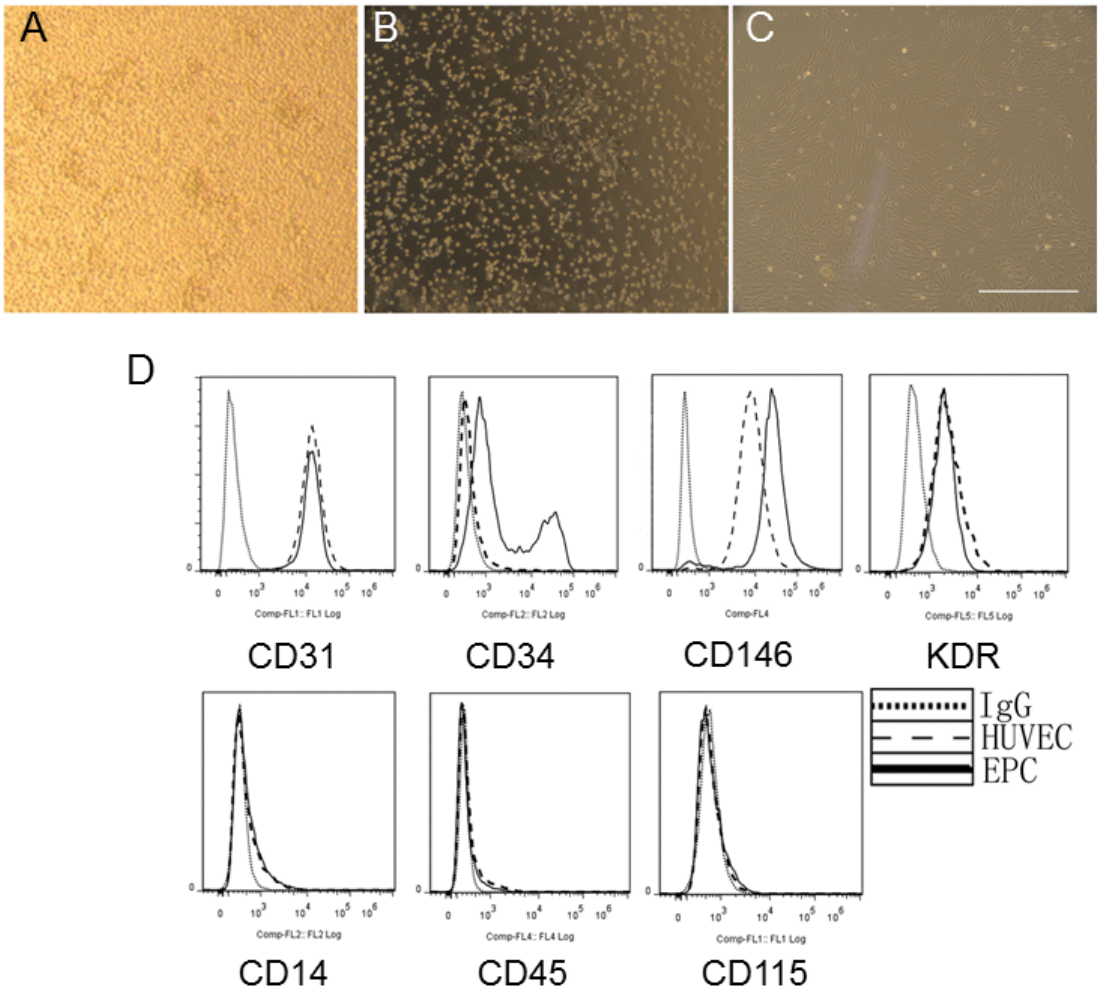

Fig. 1. Endothelial progenitor cell (EPC) development from peripheral blood mononuclear cells (PBMCs) and flow cytometric phenotyping.

4.1.2 Platelets promote EPC tube formation

The effect of platelets on the angiogenic process was evaluated using the in vitro tube formation assay on Matrigel. We found that platelets enhanced EPC tube formation in complete medium when the ratio of platelet to EPC was 200:1 (Fig. 2C).

We also determined the effect of platelets on EPC tube formation in basic medium, which had a minimal supplementation of endothelial growth factors. We found that EPCs suspended in the medium with only 0.5% FBS had a limited formation of capillary-like structures, and that platelet supplementation showed much more rapid and marked enhancement on the capillary-like network formation of EPCs (Fig. 2D). For a better elucidation of platelet-promoted tube formation, the basic medium and the EPC:platelet ratio of 200:1 were chosen for further experiments.

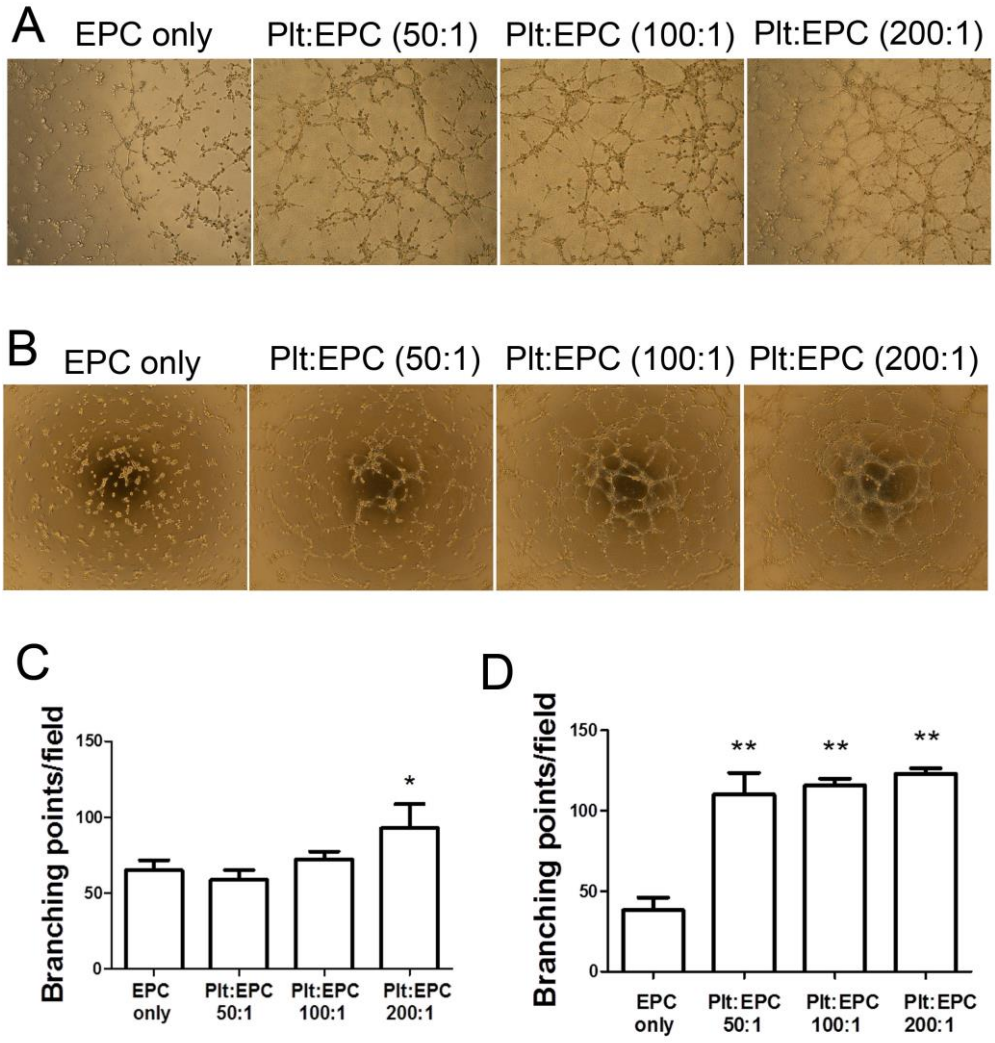

Fig. 2. Platelets promote tube formation of EPCs cultured in complete medium and basic medium.

4.1.3 Platelets surpass platelet releasate in promoting tube formation of EPCs in basal culture medium

Platelets contain a number of growth factors, which are thought to be the principle force for platelet-promoted angiogenesis. We next compared the effects of whole platelets and total platelet releasate on EPC tube formation in our system. As seen in Fig. 3A, both platelets and total platelet releasate enhanced EPC tube formation. However, platelets promoted angiogenic responses more potently than the total platelet releasate (Fig. 3B).

Since platelets, especially newly released ones, contain abundant mRNAs, platelets can undergo de novo synthesis of angiogenic proteins upon activation. To clarify if de novo protein synthesis of platelets may account for the more potent enhancements in the presence of platelets, platelets

were pre-treated with puromycin (a protein synthesis inhibitor, 100 μM, 30 min), before adding to the co-culture system. To prevent platelet activation/secretion during co-culture, PGI_2 used during platelet isolation was replaced by the PGI_2 analogue iloprost. The latter can irreversibly inhibit platelet activation. Fig. 3C shows that platelet-enhanced EPC tube formation was minimally affected by puromycin or iloprost treatment, seen as unchanged the branching point counts after puromycin or iloprost treatment (Fig. 3D). Furthermore, 2% paraformaldehyde fixed platelets were also tested for their pro-angiogenic activities. Hence, fixed platelets enhanced tube formation from 37.1±7.1/field of EPC alone to 63.9±10.1/field ($P<0.05$; n=3), which was only slightly milder than that by unfixed platelets (Fig. 3E and F).

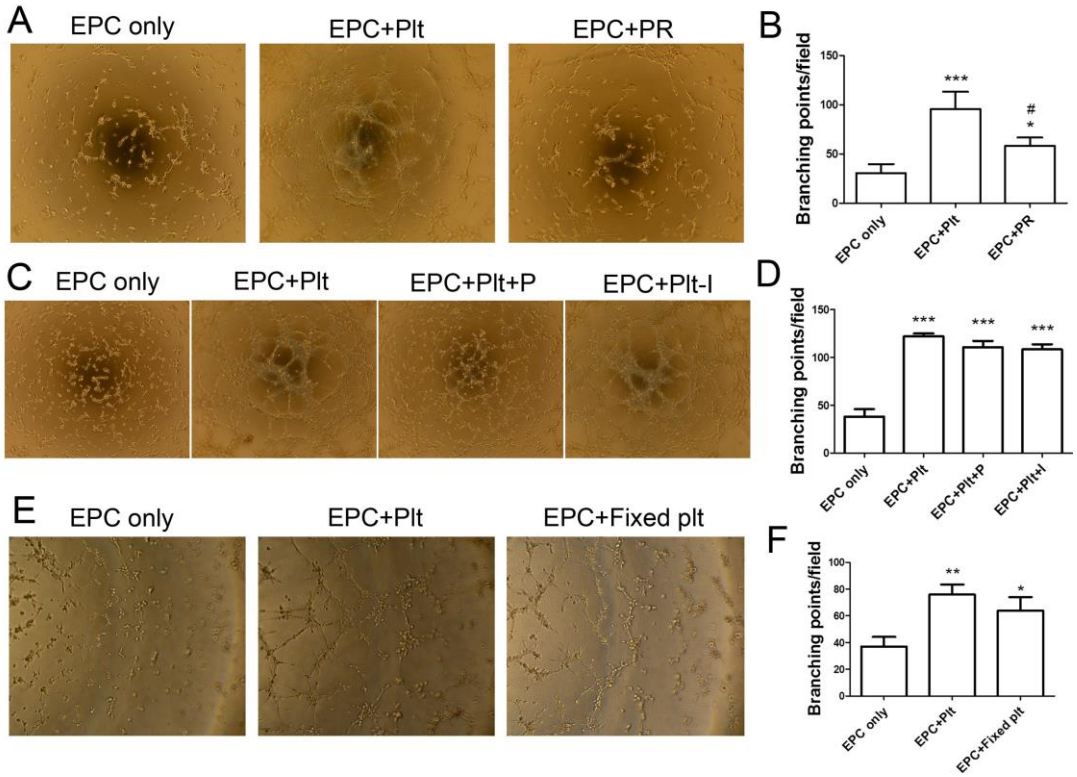

Fig. 3. Platelets surpass platelet releasate in promoting tube formation of EPCs in basic culture medium.

4.1.4 Surface glycoproteins of platelets promote EPC tube formation

Glycoproteins are the most abundant proteins on platelet membrane. To assess their possible involvements in platelet-promoted tube formation, the iloprost-treated platelets were pre-treated with neuraminidase (0.01 i.u./10^7 platelets), which strips sialic acid residues from glycoproteins. Fig. 4 shows that, as expected, supplementation of platelets significantly enhance EPC tube formation, and that neuraminidase-treated platelets failed to promote tube formation.

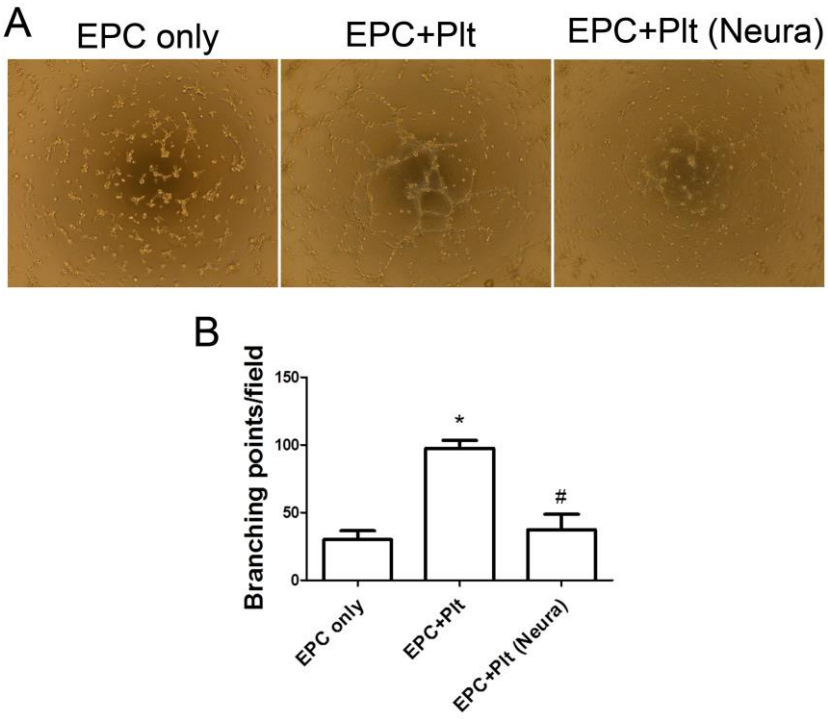

Fig. 4. Glycoproteins of the platelet membrane are important for promoting EPC tube formation.

4.1.5 CD151 in the platelets but not in EPCs is important for platelet-induced tube formation of EPCs

In order to identify platelet membrane proteins responsible for platelet pro-angiogenic effects, we employed a panel of blocking agents, including the blocking mAbs against CD151, CD31, P-selectin, and CD42b, as well as the αIIbβ3 (CD41/CD61) inhibitor SR121566. We found that blockade of platelet CD31, CD42b, P-selectin, or αIIbβ3 did not affect platelet-enhanced EPC tube formation. In contrast, CD151 blockade markedly reduced platelet-enhanced EPC tube formation, while the nonspecific IgG antibody MOPC31 had no effect (Fig. 5A).

Because both platelets and EPCs express high levels of CD151, we next compared the effects of EPC and platelet CD151 blockade on EPC tube formation. It was found that platelet pre-treatment by the CD151 blocking MAb PETA3 (clone 50-6) significantly attenuated platelet-enhanced EPC tube formation, as compared to untreated platelets. In contrast, pre-incubation of EPCs with the CD151 blocking antibody had no effect on platelet-enhanced EPC tube formation (Fig. 5C). These results further indicated that platelet-expressed CD151 accounts for platelet enhanced EPC tube formation.

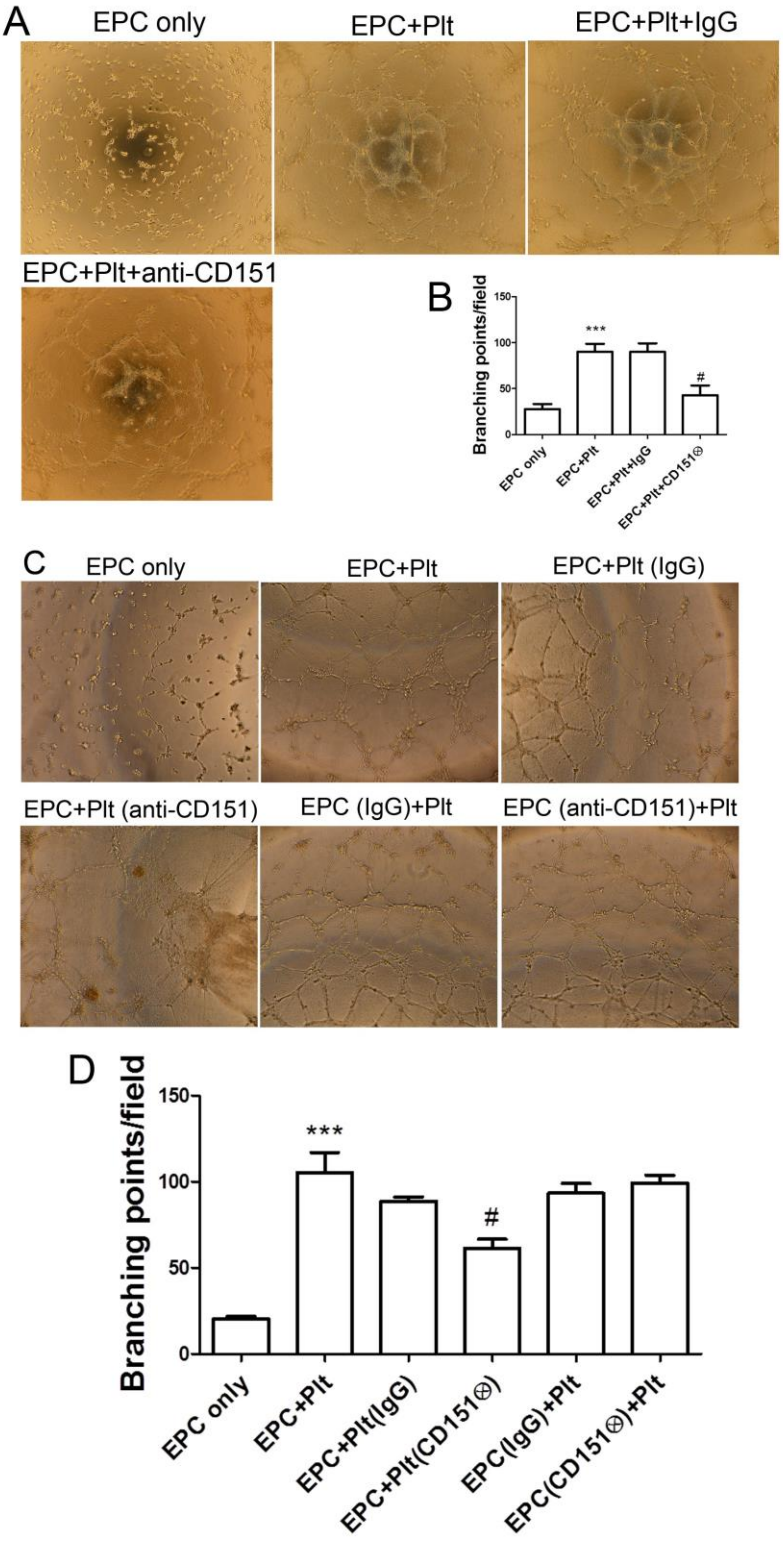

Fig. 5. CD151 on platelets but not on EPCs is important for platelet-enhanced tube formation of EPCs.

4.1.6 Integrin α6 is involved in platelet-enhanced EPC tube formation

Tetraspanins, including CD151, interact with various transmembrane proteins, such as integrins, and act as a molecular organizer that regulates the formation of functional clusters of membrane proteins. CD151 is known to link to laminin-binding integrins, such as α3β1, α6β1, α6β4, and α7β1 [233]. Therefore, the α3 blocking antibody P1B5 (20 μg/ml), the α6 blocking antibody GoH3 (20 μg/ml), and the β1 blocking antibody 13 (20 μg/ml) were added in the co-culture system. As shown in Fig. 6A, anti-α6 but not anti-α3 MAb decreased platelet-enhanced EPC tube formation, while the anti-β1 antibody totally abolished EPC tube formation on matrigel. In contrast, RGDS (Arg-Gly-Asp-Ser) peptide, which recognises the RGD motifs in the integrins and inhibits integrin receptor function, had no effect on platelet-enhanced EPC tube formation (Fig. 6C).

Platelets express the laminin-binding integrin α6β1 [234, 235], and EPCs express both α6β1 and α6β4 [236]. Hence, we next compared the effects of EPC and platelet integrin α6 blockade on platelet-enhanced tube formation by pre-incubation of the α6 blocking antibody GoH3. Fig. 6C shows that either platelet α6 integrin blockade or EPC α6 integrin blockade similarly reduced tube formation. Moreover, simultaneous inhibition of both platelet and EPC α6 integrins showed no deference in platelet-enhanced EPC tube formation. These results suggested that integrin α6 was important for platelet-promoted EPC tube formation.

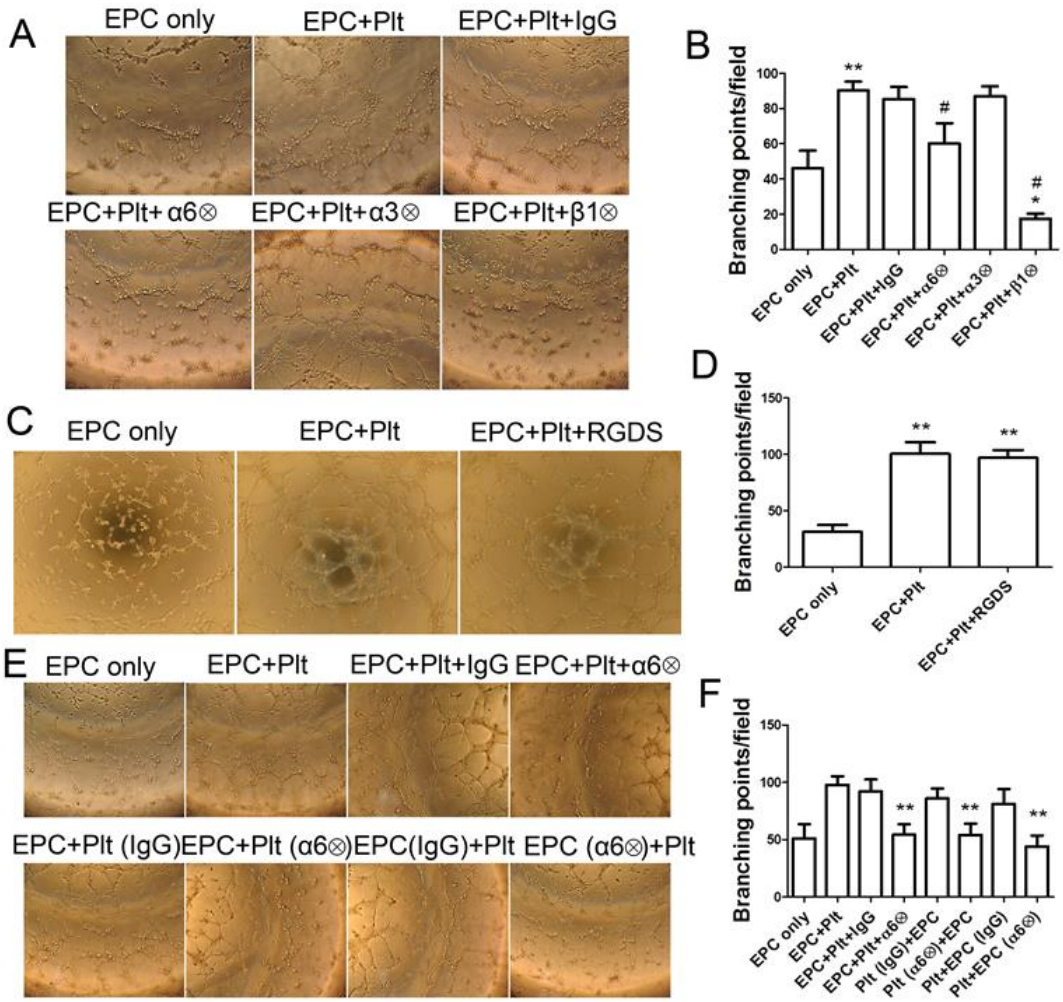

Fig.6. Integrin α6β1 is involved in CD151-promoted EPC tube formation.

4.1.7 Platelets increase EPC tube formation through the Src and PI3K pathways

To further study platelet mediated EPC angiognic responses, the potential involvements of different signalling pathways were also investigated. Thus, EPCs were pre-treated with the Src kinase inhibitor PP2 (10 μM), the PI3K inhibitor LY294002 (25 μM), or the pan PKC inhibitor Ro318220 (10 μM) for 20 min on ice prior to tube formation assays. Fig. 7 shows that PP2 and LY294002 effectively inhibited platelet-mediated EPC tube formation, which almost abolished the tube formation induced by platelets. Ro318220 mildly attenuated EPC tube formation, which produced an approximately 50% inhibition.

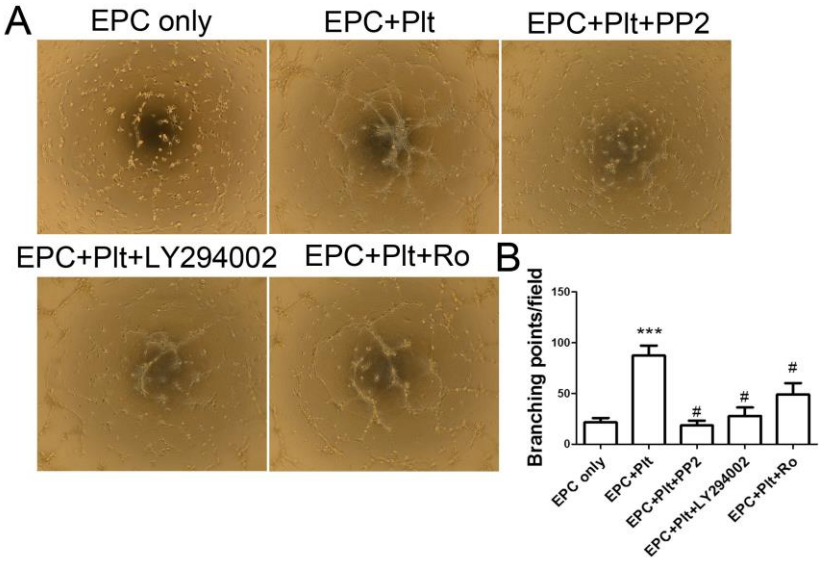

Fig. 7. Platelets-promoted tube formation involves Src-PI3K signaling of EPCs.

In summary, platelets can promote angiogenic activities of EPCs independently from platelet-released mediators. Platelets exert the enhancement via platelet membrane-expressed tetraspanin CD151 that promotes EPC tube formation through interaction with α6 integrins and via the Src-PI3K signalling pathways in EPCs. Together with platelet-released angiogenic regulators, platelet membrane components constitute the optimal pro-angiogenic effects of platelets, and may serve as a useful target for intervention of platelet angiogenic activities.

4.2 DISTINCT PLATELET PACKAGING AND RELEASE OF PROANGIOGENIC AND ANTIANGIOGENIC FACTORS ON DIFFERENT PLATELET STIMULI (PAPER II)

Platelets contain both pro- and anti-angiogenic factors. A recent study showed that some pro-angiogenic and anti-angiogenic factors are stored in separate α-granules, and that thrombin receptor PAR1 and PAR4 stimulation induce distinct release of pro- and anti-angiogenic factors [63]. Here, we wanted to clarify if distinct packaging of platelet pro- and anti-angiogenic factors is a general phenomenon and if and how platelet agonists other than thrombin also induce a selective secretion of pro- and anti-angiogenic factors.

We detected the localization of angiogenic factors within the platelet α-granules by immunofluorescent staining. We found that granules were mobilized toward cell periphery, often seen in a ring-like staining pattern (arrows) after stimulation (Fig. 8Ai, Bi, Ci, Di). Double immunofluorescence labeling demonstrated that most SDF-1α (red) and PF4 staining (green) was in separate α-granules, and little colocalization in yellow (arrowheads) was seen in either resting (Fig. 8Eiii) or ADP-stimulated platelets (Fig. 8Fiii). We confirmed the presence of distinct populations of α-granules in human platelets at the ultra-structural level using immuno-electron microscopy. As expected, SDF-1α (Fig. 8G) or PF4-probing gold particles (Fig. 8H) only present in a subpopulation of α-granules. Double immunogold microscopy confirmed that only some α-granules contain both SDF-1α (arrows) and PF4 (arrowheads) gold particles (Fig. 8I), supporting our findings with confocal microscopy and the notions that the counteracting factors are mostly segregated in separate α-granules and that different α-granules are packed with different proteins.

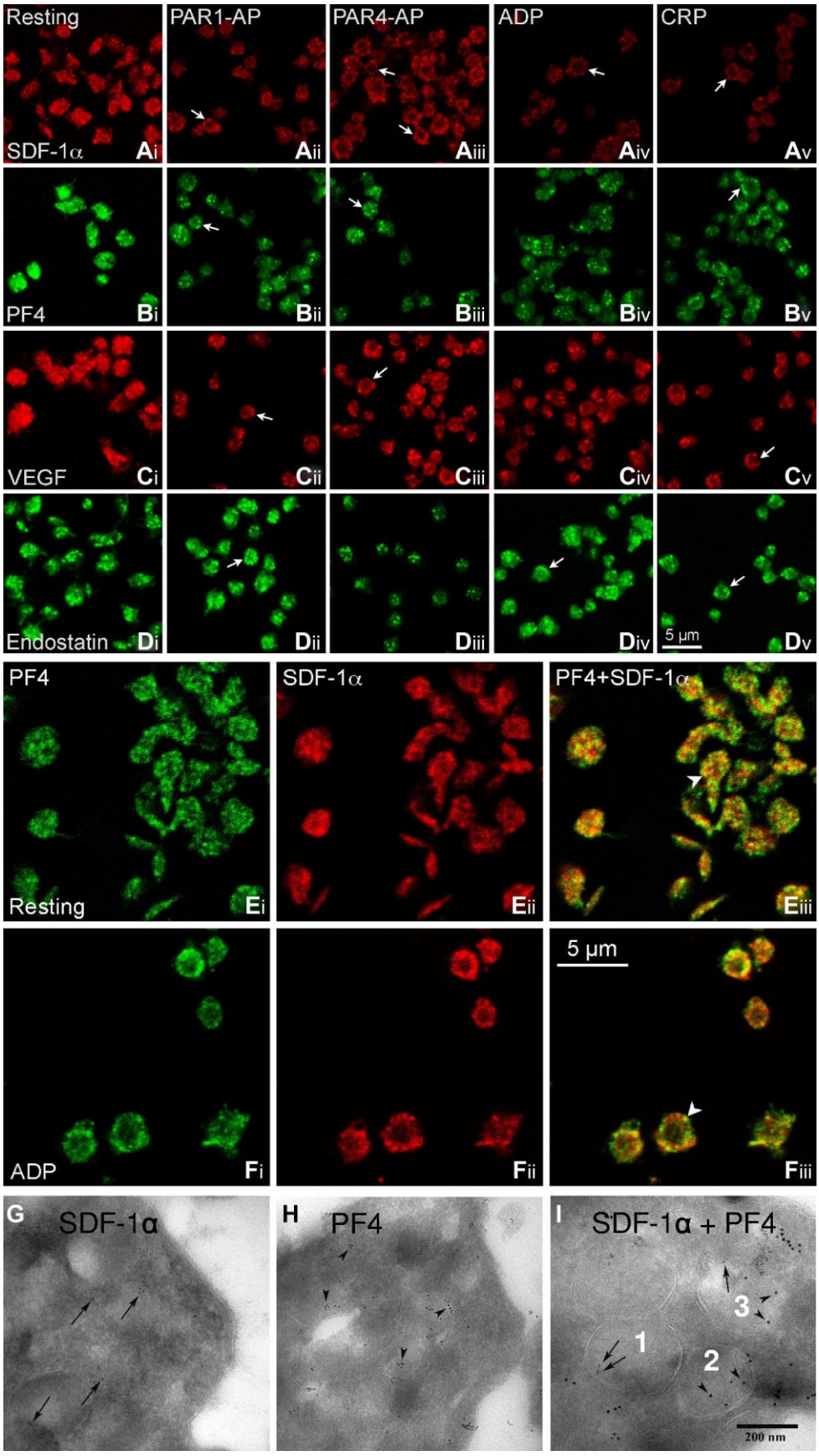

Fig. 8. Platelets store proangiogenic and antiangiogenic regulators in separate α-granules.

4.2.1 Distinct secretion and surface expression of platelet proangiogenic and antiangiogenic factors

We next detected the levels of the angiogenic factors in supernatant by ELISA to determine whether platelet activation evokes distinct secretion of angiogenic factors. We found that stimulation with PAR1-AP, ADP, and CRP (targeting GPVI) induced massive release of SDF-1α and VEGF, as evidenced by significant elevations of SDF-1α (Fig. 9A) and VEGF levels (Fig. 9B) in the supernatant of washed platelets. These agonists induced, however, much less release of the antiangiogenic PF4 (Fig. 9C) or actually no release of endostatin (Fig. 9D). The data indicate that PAR1-AP, ADP, and CRP selectively enhance proangiogenic factor release. We all know ADP has two receptors on platelets, P2Y1 and P2Y12. To identify which receptor account for ADP-induced angiogenic factors release, we activated platelets with ADP in presence of vehicle or P2Y12 antagonist. We found the combination of ADP and a P2Y12 antagonist showed that ADP-induced VEGF and PF4 release was mainly via P2Y12 activation, whereas ADP-induced SDF-1α secretion was primarily via P2Y1 receptor engagement. In contrast, PAR4-AP triggered marked release of PF4 (Fig. 9C) and endostatin (Fig. 9D) but limited release of SDF-1α (Fig. 9A) and VEGF (Fig. 9B). Thus, PAR4-AP stimulation promotes a selective release of platelet antiangiogenic factors. Furthermore, platelet secretion of angiogenic factors seems to be a rapid process. The maximal PAR1-stimulated VEGF release was reached in less than 5 minutes, with VEGF levels increased from undetectable of resting platelets to 49.8 ±1.8, 50.6 ±0.6, and 49.9 ±0.6 pg/mL at 5, 15, and 30 minutes of stimulation, respectively. Similarly, PAR4-stimulated endostatin release peaked at 5 minutes (89.1 ±10.6 vs 0.9 ±0.1 ng/mL of resting platelets, and 81.3 ±11.9 ng/mL after 30 minutes stimulation; n=3).

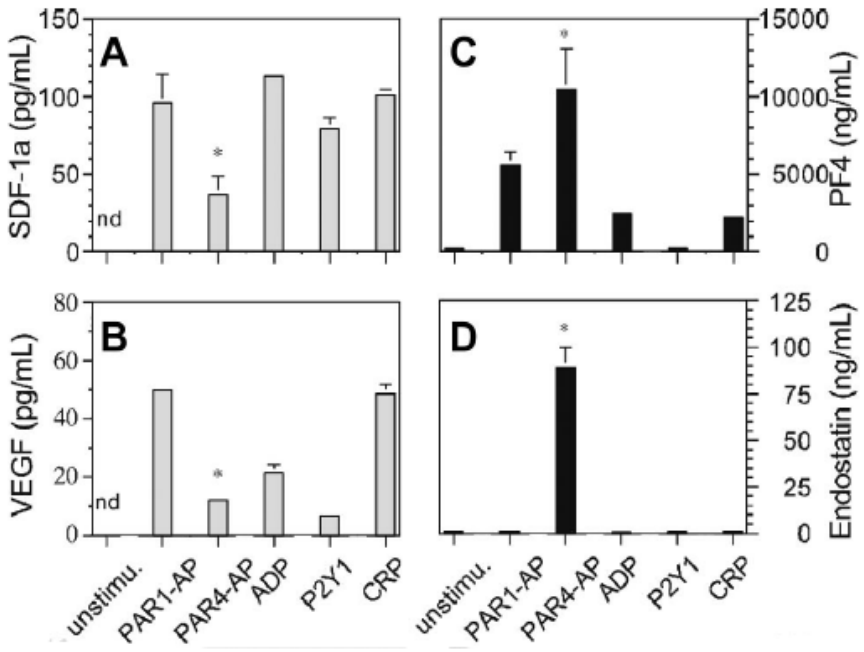

Fig. 9. Distinct secretion of platelet proangiogenic and antiangiogenic factors.

In summary, proangiogenic factors and antiangiogenic factors are mostly packing in distinct populations of α-granules in platelets, and different platelet stimuli evoke distinct secretion of proangiogenic and antiangiogenic factors. PAR1, ADP, and GPVI stimulation favors proangiogenic, whereas PAR4 promotes antiangiogenic, factor release.

4.3 PAR1-STIMULATED PLATELET RELEASATE PROMOTES ANGIOGENIC ACTIVITIES OF ENDOTHELIAL PROGENITOR CELLS MORE POTENTLY THAN PAR4-STIMULATED PLATELET RELEASATE (PAPER III)

4.3.1 Neither PAR1-PR nor PAR4-PR has any effect on EPC proliferation, cell cycle or apoptosis

Given that PAR1 and PAR4 stimuli induce selective platelet release of proangiogenic and antiangiogenic factors. Our follow-up study was thus to investigate if PAR1-stimulated platelet releasate (PAR1-PR) and PAR4-PR regulate angiogenic properties of EPCs in different manners.

We investigated the influence of platelet releasates on EPC proliferation. We found that supplementation of platelet releasates to cultured EPCs did not alter cell proliferation of cultured EPCs (Fig. 10A). There was no difference between PAR1-PR and PAR4-PR or among the platelet releasate concentrations of 2.5%, 5%, and 10%. To clarify these surprising results, EPC culture with platelet releasates was prolonged to 48 h. The releasates were again found to have no effect on EPC proliferation (Fig. 10B). To further elucidate the potential influences of platelet releasates on EPC proliferation, we monitored cell cycle and apoptosis. Fig. 10C, D show that neither 10% PAR1-PR nor 10% PAR4-PR significantly influenced EPC cell cycle distribution in the G0/ G1, S, or G2/M phase. Fig. 10E, F show that addition of either PAR1-PR or PAR4-PR to cultured EPCs did not alter the percentages of the total Annexin V^+ EPCs, which included early and late apoptotic cells, as well as necrotic cells.

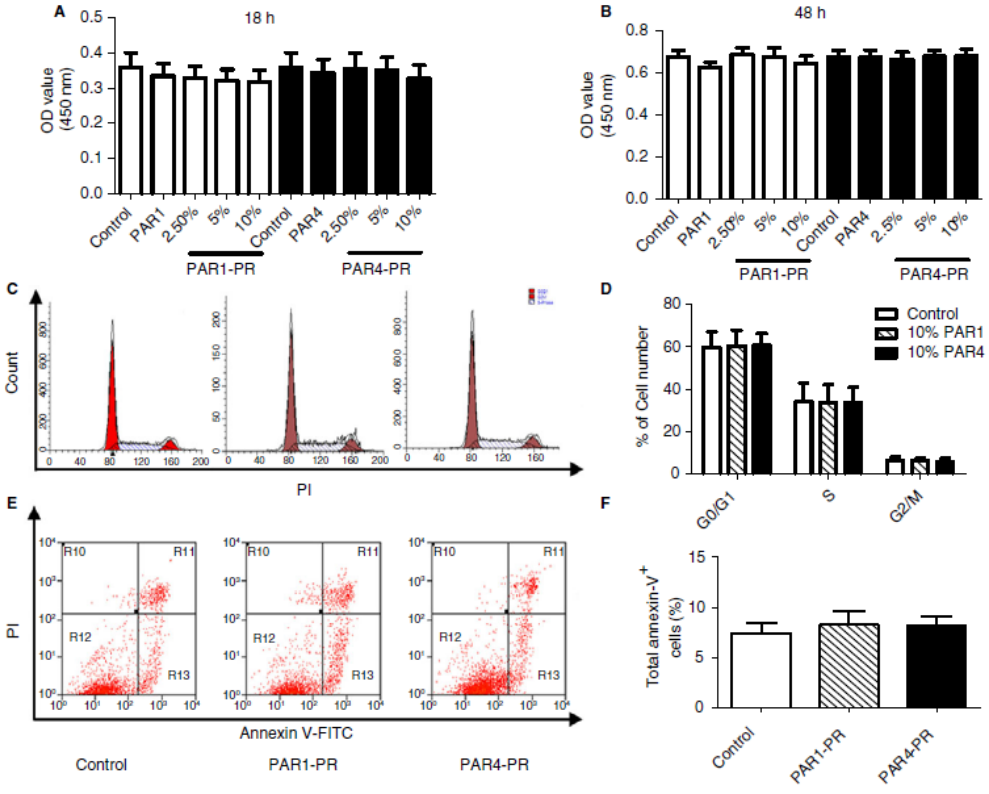

Fig. 10. Influences of platelet releasates on EPC proliferation, cell cycle, and apoptosis.

4.3.2 Both PAR1-PR and PAR4-PR enhance EPC migration and tube formation

We next investigated the effect of platelet releasates on EPC migration by using a Boyden chamber chemotactic migration assay. After a 6-h culture, we found that the numbers of migrated EPCs were increased in the wells containing PAR1-PR (relative increase by 25%, compared with complete medium alone) and PAR4-PR (29%; $P < 0.05$ for both; $n = 4$) but not PAR1-AP or PAR4-AP (Fig. 11A). As the incubation time increased to 24 h, the numbers of the migrated cells were further increased, with a relative enhancement by 44% in PAR1-PR–containing wells and by 40% in PAR4-PR–containing wells ($P < 0.05$ for both; $n = 6$) (Fig. 11B). Moreover, there was no difference between the treatments with PAR1-PR and PAR4-PR on either observation time points.

We also examined the effects of platelet releasates on EPC tube formation with an in vitro Matrigel assay. We found both PAR1-PR and PAR4-PR markedly enhanced tube formation of the EPCs, seen as increased densities of the capillary network in the complete medium (Fig. 11E). When capillary network branch points were counted, PAR1-PR and PAR4-PR both increased the branch numbers of capillary networks, compared with those without platelet releasates (Fig. 11F).

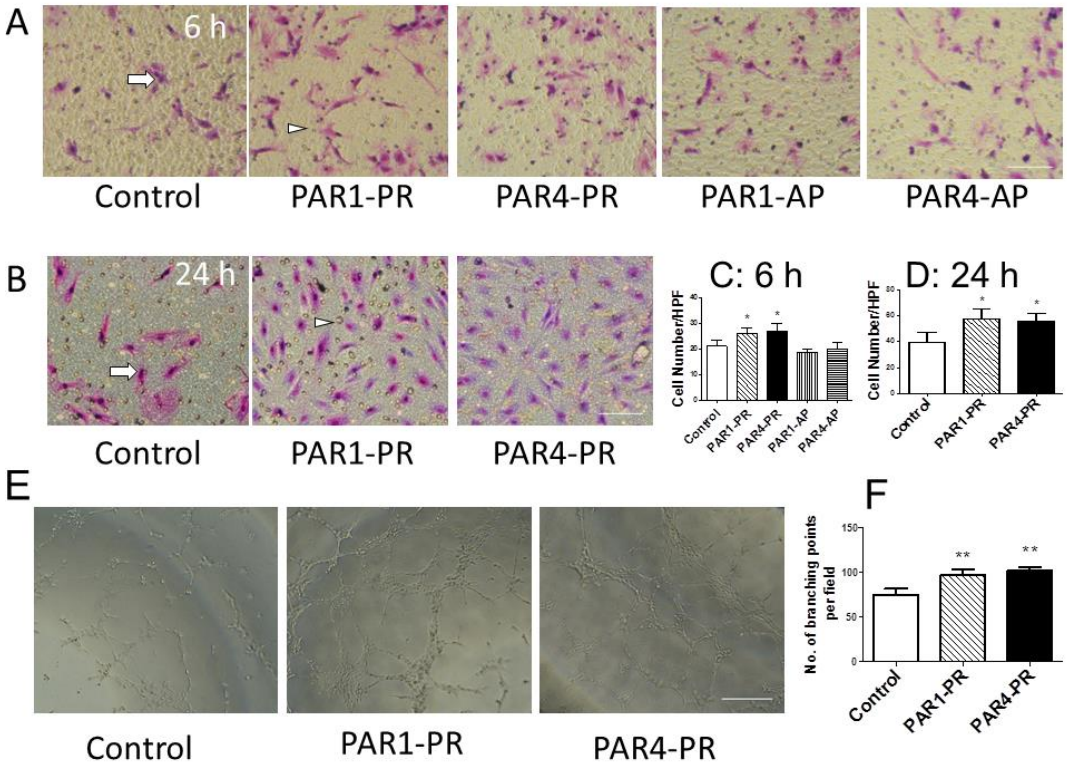

Fig. 11. Platelet releasates enhance EPC migration and tube formation.

4.3.3 Impact of platelet-released VEGF, MMP, and SDF-1α on EPC migration and tube formation

Activated platelets release a number of angiogenic regulators, such as VEGF, MMP, and SDF-1α, which can enhance EC migration and/or tube formation. We therefore investigated whether these factors were involved in the enhancements of platelet releasates on EPC angiogenic responses by using corresponding inhibitors. To more clearly elucidate the effects of platelet releasates on EPC migration, we chose to use the EBM-2 medium containing 10% FBS but not the growth factor cocktail of SingleQuots Kit as the chemotactic source in the migration experiments. Fig. 12 shows that, in the absence of platelet releasates (open bars), VEGF receptor blockade by Ki8751 (10 μM) did not reduce EPC migration toward FBS-containing medium in the lower chamber. MMP inhibition by GM 6001 (20 μM) and SDF-1α/CXCR4 inhibition by AMD3100 (10 μM) were associated with lower numbers of migrated EPCs but without a statistical significance. The migration of EPCs was significantly increased in the presence of 10% PAR1-PR and 10% PAR4-PR ($P < 0.05$ for both; $n = 4$) in a similar manner. The enhancements were reduced by MMP inhibition and SDF-1α/CXCR4 inhibition ($P < 0.05$ for all).

Thus, we next chose to use EPCs suspended in the medium containing only 0.5% FBS in the tube formation experiments to clearly demonstrate the effects of platelet-released angiogenic regulators on EPC tube formation. In this system, EPCs suspended in the basal medium had a limited formation of capillary-like structures after 6-h culture (Fig. 12C; with branch numbers at 33.5 ± 5.4 per field). Supplementation of 1 μM PAR1-AP (31.2 ± 9.0 per field) or 10 μM PAR4-AP (34.0 ± 7.5 per field), which corresponded to their levels in 10% platelet releasates, had no effects on EPC capillary network formation. However, both PAR1-PR and PAR4-PR significantly enhanced the capillary-like network formation of EPCs (Fig. 12C, D). More profound enhancement was seen with PAR1-PR that increased the branch number to 60.7 ± 9.3 per field (representing the relative increase of 80.3%; $P < 0.05$). Fig. 12D also shows that PAR1-PR– and PAR4-AP– enhanced EPC tube formations were markedly inhibited in the presence of an inhibitor of either VEGFR2, MMP, or SDF-1α. Interestingly, each of those inhibitory agents virtually abolished the enhancing effects of PAR1-PR or PAR4-PR (Fig. 12). These data may suggest that platelet releasate–enhanced EPC tube formation is exerted as a cooperation of multiple platelet-derived angiogenic regulators.

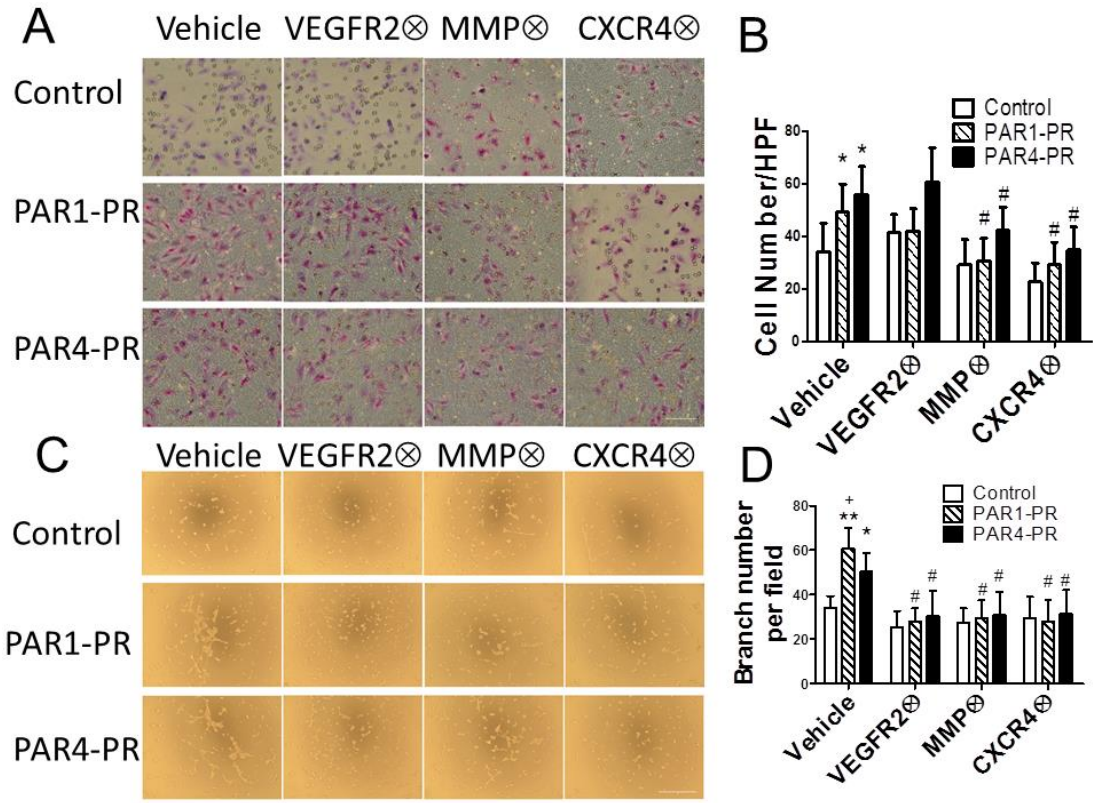

Fig. 12. Effects of VEGF, MMP, or SDF-1α inhibition on platelet releasate–enhanced EPC migration and tube formation.

4.3.4 PAR1-PR promotes stronger vasculogenesis in vivo than PAR4-PR

Using a murine model of vasculogenesis in vivo with the implantations of Matrigel containing 10% platelet releasates, Fig. 13A shows that 2-week implantation of Matrigel plugs containing supernatant of unstimulated platelets did not induce marked new vessel formation. However, Matrigel plugs containing PAR1-PR induced significant vasculogenesis, and the new vessels were much better developed, as evidenced by a more intense branching of the new vessels (Fig. 13B). In contrast, Matrigel plugs containing PAR4-PR induced considerably less vasculogenesis, and the new vessels were less well developed and with limited branches (Fig. 13C), compared with these in PAR1-PR plugs. When branch numbers of newly formed vessels were counted, both PAR1-PR and PAR4-PR induced significantly more vasculogenesis than did Rest-PR, while PAR1-PR–induced new vessel formation was much more marked than those by PAR4-PR (Fig. 13D). Immunofluorescent staining of the endothelium-specific marker CD31 confirmed that there was more intense vasculogenesis in PAR1-PR Matrigel plugs (Fig. 13F) than in PAR4-PR (Fig. 13G) or Rest-PR Matrigel plugs (Fig. 13E). The H&E staining of paraffin-embedded Matrigel plug sections also revealed that new vessel formation was rare in Rest-PR plugs (Fig. 13I), most intense and well developed in PAR1-PR plugs (Fig. 13J), and thinly scattered in PAR4-PR plugs (Fig. 13K). Moreover, PAR1-PR and PAR4-PR induced a more intense cell infiltration, likely blood-borne inflammatory cells, into the plugs.

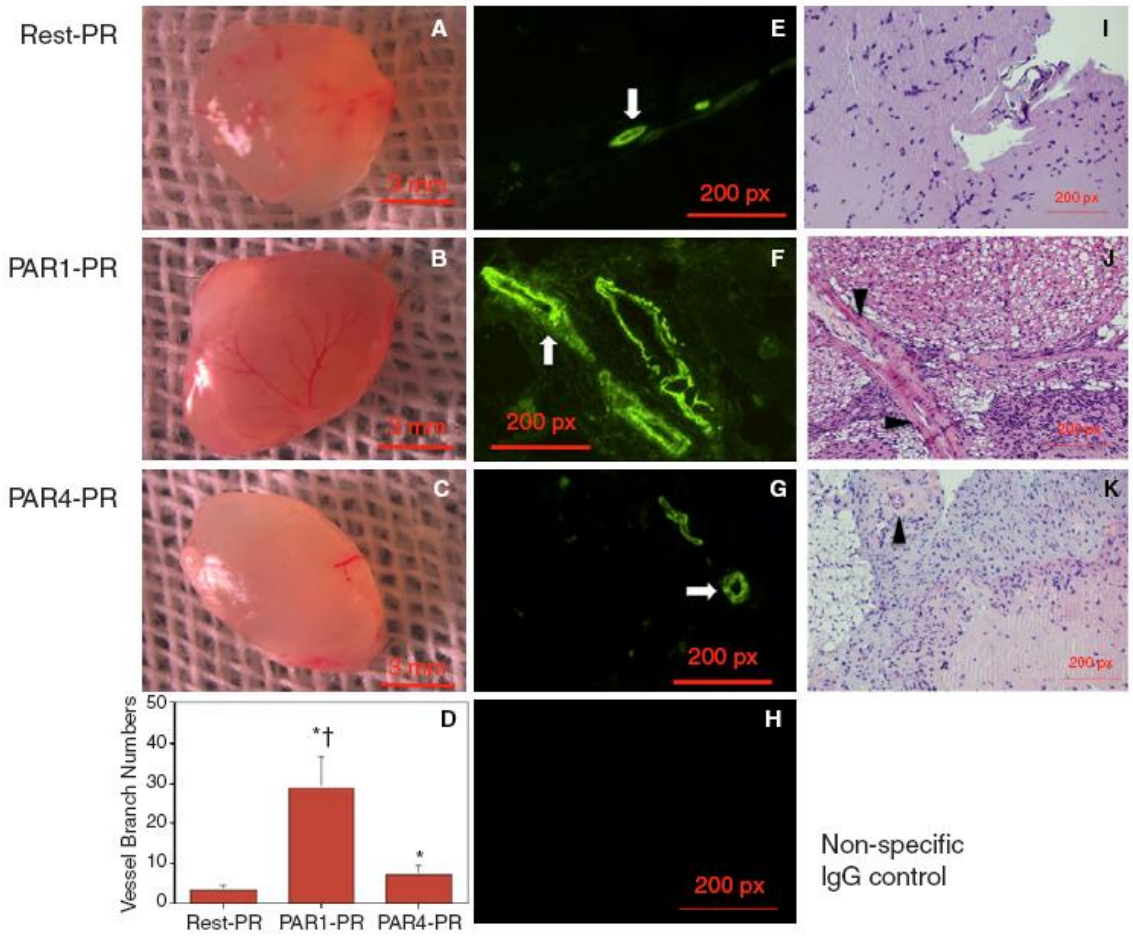

Fig. 13. PAR1-PR promotes stronger vasculogenesis in vivo than PAR4-PR.

In summary, the platelet releasates from both PAR1- and PAR4-stimulated platelets enhanced EPC migration and tube formation, but had no influence on EPC proliferation. However, PAR1-PR–enhanced capillary network formation of EPCs was more profound, and the enhancement was even more evident in the mouse model of Matrigel implantation. The enhancements involve multiple factors, as intervention of either VEGF, SDF-1α, or MMP abolished platelet releasate–enhanced tube formation of EPCs.

4.4 THROMBIN INDUCES DE NOVO PROTEIN SYNTHESIS OF ANGIOGENIC FACTOR IN HUMAN PLATELETS (PAPER IV)

4.4.1 Thrombin stimulation induces de novo protein synthesis of SDF-1α in human platelets

Albeit an anuclear cell, platelets can actively synthesize proteins closely related to their functions, which is a newly recognized but yet important feature of "late" platelet activation events [104]. Hence, it is interesting to reveal if platelet activation initiates protein synthesis of angiogenic regulators.

We isolated leukocyte-depleted washed platelet, and activated with thrombin. We found that the intensity of SDF-1α-immunoreactive bands were decreased after thrombin stimulation for 30 min, indicating reduced levels of SDF-1α in platelets, i.e. SDF-1α release upon stimulation. However the SDF-1a immunoblotting intensity was partially restored after 16 h of culture, suggesting de novo protein synthesis of SDF-1α. As expected, the immunoblotting intensity of the loading control glyceraldehyde-3-phosphate dehydrogenase remained largely unchanged throughout the experiments. When SDF-1α immunoblotting intensity was quantified, SDF-1α levels were significantly decreased by 30 min of thrombin stimulation, and showed a rebound after 16 h, albeit to a level lower than that before stimulation (Fig. 14C). Similar to that of SDF-1α, thrombin stimulation markedly reduced the intensity of angiostatin-immunoreactive bands (Fig. 14B,D), indicating that thrombin also induced platelet secretion of angiostatin. In contrast to the rebound seen with SDF-1α, the reduction in platelet angiostatin content remained after 16 h of cell culture.

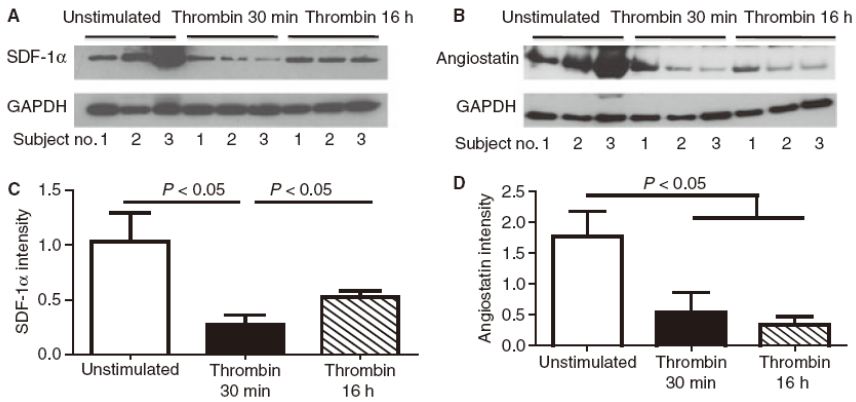

Fig. 14.Thrombin stimulation induces de novo protein synthesis of SDF-1α in human platelets.

4.4.2 Thrombin stimulation induces SDF-1α mRNA maturation in human platelets

Next we investigated whether changes in platelet protein content were related to mRNA maturation. To demonstrate the expression of functional SDF-1α mRNA, we chose a quantitative real-time RT-PCR (qRTPCR) with primers spanning an exon–exon junction of SDF-1a/CXCL12, which allowed measurement of mature mRNA of SDF-1α but not of pre-mRNA; 18S rRNA was used as an endogenous control. The cDNA synthesis was performed with 200 ng of total RNA and a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Fig. 15A shows that qRT-PCR readily detected 18S rRNA in both unstimulated and thrombin-activated platelets. For SDF-1α, however, no mature mRNA was detected in unstimulated platelets, whereas significant amplification of mature SDF-1α mRNA was found in thrombin-activated platelets after approximately 30 PCR cycles. These results suggest that thrombin stimulation induced maturation of SDF-1α mRNA, and that the mRNA was expressed at a relatively low level as compared with 18S rRNA. When the angiostatin/plasminogen (PLG) qRT-PCR assay was used, no significant angiostatin/PLG mRNA was detected in either unstimulated or thrombin-stimulated platelets (Fig. 15B).

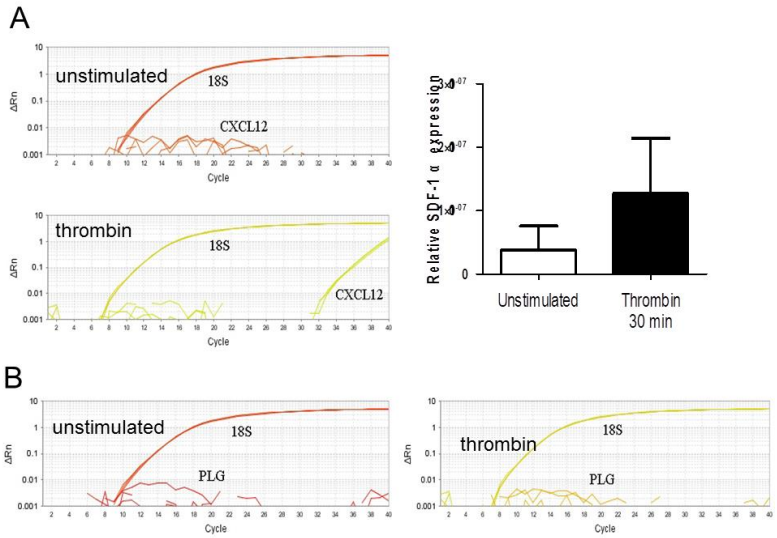

Fig. 15.Thrombin stimulation induces mRNA maturation of SDF-1α in human platelets.

In conclusion, platelets contain SDF-1α mRNA transcripts. Thrombin stimulation induces SDF-1α mRNA maturation, which leads to de novo synthesis of SDF-1α after activation. The newly synthesized SDF-1a may reinforce platelet angiogenic activities in remodelling and repair of the injured vessels.

5 GENERAL DISCUSSION

The present thesis work provides new evidence supporting that platelets are an important player in angiogenesis. We have shown that platelets store pro-angiogenic and anti-angiogenic regulators in separate α–granules, and may release them distinctly upon different platelet stimuli. Distinct releases of platelet angiogenic regulators display distinct impacts on angiogenesis. PAR1-PR, which is rich in pro-angiogenic regulators, promotes angiogenesis more profoundly both in vitro and in vivo as compared to PAR1-PR, which is prone anti-angiogenic regulator release. Apart from platelet-released mediators, we have also identified that platelet membrane components tetraspanin CD151 and α6β1 integrin, as well as EPC α6β1 integrin are important for platelet-enhanced EPC tube formation. Furthermore, our results indicate that platelets undergo de novo protein synthesis of SDF-1α upon activation.

During the last decades, accumulating evidence indicates that platelets are closely involved in angiogenesis [13, 17]. Investigators have identified numerous pro- and anti-angiogenic factors that are stored in platelets and released after platelet activation. The pro-angiogenic factors found in platelets include VEGF, PDGF, bFGF, IGF-1, S1P, and MMPs. The anti-angiogenic factors found in platelets include TSP-1, PF4, PAI-1, and angiostatin [13, 17]. In most of the experiments, platelets play a proangiogenic effect. However, to what extent that pro-angiogenic properties of platelets are attributed to platelet-release angiogenic factors is an issue to be clarified. Some papers suggest that the growth factors released from platelets are not enough for the effect. For instance, Pipili-Synetos et al. [231] found that platelets, but not supernatant of matrigel-activated platelets or thrombin-activated platelets, promoted EC tube formation. Kent et al. [237] also showed that attachment of fresh platelets, but not addition of platelet releasate, stimulated EC proliferation in an organ culture model of arterial injury. In contrast, there are also papers showing that ADP-induced platelet releasate and thrombin-induced platelet releasate increase EC tube formation and angiogenesis in vivo [66, 69, 238]. The major difference among these contradicting papers is that the concentration of platelets used. The studies with negative results used the releasates from 1-2 $\times 10^7$/ml platelets [69, 231], whilst the reports with positive results were using platelet releasates from at least 10 times higher concentrations of platelets (2-4 $\times 10^8$/ml) [69, 238] The present thesis work showed that 10% platelet releasates from 2 $\times 10^9$/ml increased EPC migration, tube formation, and vasculogenesis in vivo. Thus, platelet releasates from physiological concentrations of platelets are able to modulate EC and EPC angiogenesis.

We also found that platelets had a higher enhancement for angiogenesis than the total platelet

releasate, which was consistent with previous paper that the angiogenic responses to platelets were more pronounced than that of the platelet releasate [69]. Glycoproteins are the most abundant proteins on platelets, and have been suggested to be involved in platelet-mediated angiogenesis [231]. Hence, we have demonstrated that platelet membrane glycoproteins contribute importantly to platelet-enhanced angiogenic activities of EPCs, because neuraminidase treatment abolished platelet-enhanced angiogenesis. We have further identified that platelet membrane-expressed tetraspanin CD151 is responsible for the enhancement, and demonstrated that CD151 promotes EPC tube formation through interaction with α6 integrins and via the Src-PI3K signalling pathways of EPCs. Interestingly, it seems that integrin α6 on both EPC and platelet membranes are engaged in the action, because either EPC or platelet integrin α6 blockade showed an identical effect in attenuating platelet-enhanced EPC tube formation. EPC α6 integrin may interact with laminin-111 present in Matrigel and thereby mediate the angiogenesis, as recently demonstrated by Bouvard et al. [239]. Platelet α6β1 integrin may also interact with the Matrigel laminin-111 or, alternatively, with cell-associated laminins produced by EPCs.

Platelets contain both pro- and anti-angiogenic factors and release them after activation. Recent studies indicate that platelets may store pro- and anti-angiogenic regulators in separate α-granules, and release differentially upon different stimuli. Ma et al. [62] first demonstrated that the thrombin receptor PAR1 and PAR4 stimulation induce differential releases of pro- and anti-angiogenic regulators. PAR1-specific stimulation induces VEGF release, whereas PAR4 activation resultes in endostatin release. Subsequently, Italiano et al. [63] further revealed that VEGF and endostatin are stored into separate α-granules in both platelets and megakaryocytes. In line with these findings, paper II of the present thesis showed that platelets mostly store proangiogenic and antiangiogenic regulators in separate α-granules, and that platelets selectively release proangiogenic or antiangiogenic regulators upon different stimuli [66].

However, there is also evidence showing that platelet angiogenic regulators may be randomly packed into platelet α-granules but released with a distinct protein cluster, and that the distinct release may depend on activation intensity and secretion kinetics of platelets. Van Nispen tot Pannerden et al. [240] identified two different types of α-granules, spherical and tubular α-granules. They also noted that those tubular granules contain fibrinogen but not VWF, and those spherical α-granules, on the other hand, contain both fibrinogen and vWF. However, tubular granules were identified in only proximately 16% of the platelets. Kamykowski et al. [64] failed to show evidence for coclustering of angiogenic regulators into functionally distinct α-granule

populations using a super-resolution analysis of 15 different human α-granule proteins and quantified 28 different pair-wise comparisons. Jonnalagadda et al. [65] further performed a systematic quantification of granule secretion using microenzyme-linked immunosorbent assay arrays for 28 distinct α-granule cargo molecules in response to four different agonists, and showed that there were no obvious functional patterns to PAR1- or PAR4-stimulation. They found that PAR4-AP did not induce release of as many different molecules as did thrombin, convulxin, or PAR1-AP. More recently, van Holten et al. [241] showed that the most abundant α-granule proteins were released in similar quantities from platelets after maximum stimulation with either PAR-1 or PAR-4 using the mass spectrometry based quantitative proteomics.

Although distinct releases of platelet angiogenic regulators upon different stimuli are still under a debate, it has been reported that different platelet releasates may exert counteracting effects on angiogenesis [66]. Thus, ADP-induced platelet releasate promoted EC tube formation, whereas TXA2-induced platelet releasate inhibited EC tube formation [66]. However, recently, Etulain et al. [242] showed that both PAR1-PR and PAR4-PR promoted angiogenesis in a similar pattern. In paper III, we found that platelet releasates by the thrombin receptor PAR1 and PAR4 stimulation only result in different degrees of enhancements on angiogenesis. Our results demonstrated that intervention of VEGF, SDF-1α, or MMP each abolished the enhancements of EPC tube formation by platelet releasates. Our findings suggest that pro-angiogenic effects of platelet releasates require a cooperation of multiple angiogenic regulators. Moreover, our data indicated that the selective release of platelet angiogenic regulators mainly concerns the different release levels of platelet angiogenic regulators, and that the absolutely selective release is seen only with endostatin secretion triggered by PAR4 stimulation. Platelets release numerous angiogenic regulators after stimulation via PAR1 or PAR4, and the overall outcome of proangiogenic effects seem depend on the negotiation of all factors in the platelet releasates.

The presence of mRNA in platelets was detected more than two decades ago. However, we are only beginning to understand the roles of mRNA in platelet biology and human diseases, because platelet mRNA levels are low and the principal functions of platelets in haemostasis and thrombosis mainly concern rapid processes of platelet activation. It is now recognized that platelets are also closely involved in nonhaemostatic processes, such as inflammation and angiogenesis, which are chronic processes. There is also growing evidence that platelet mRNA expression patterns are altered in human disease [243].

Since the discovery of synthesis of Bcl-3 by activated platelets [244], platelets are found to

synthesize more and more proteins, such as TSP-1, IL-1β, PAI-1, and TF [104]. These findings also reveal that, similar to other cells, protein syntheses of platelets are preceded by signal-dependent pre-mRNA splicing. This process yields mature transcripts that are translated into precursor (IL-1β) and active (TF) proteins [105-108]. However, if platelet activation initiates protein synthesis of angiogenic regulators has not been thoroughly investigated. In paper IV, we found that thrombin stimulation induced de novo protein synthesis of pro-angiogenic SDF-1α, but not anti-angiogenic angiostatin [245]. However, the impact of the do novo synthesized protein on angiogenesis and/or vessel remodelling still needs further investigations.

6 CONCLUSIONS AND SUMMARY

Platelets can promote angiogenic activities of EPCs through membrane components. Platelets exert the enhancement via platelet membrane-expressed tetraspanin CD151 that promotes EPC tube formation through interaction with α6 integrins and via the Src-PI3K signalling pathways in EPCs. Together with platelet-released angiogenic regulators, platelet membrane components constitute the optimal pro-angiogenic effects of platelets, and may serve as a useful target for intervention of platelet angiogenic activities.

Platelets contain both pro- and anti-angiogenic factors. The proangiogenic factors and antiangiogenic factors are mostly packing in distinct populations of α-granules in platelets, and different platelet stimuli evoke distinct secretion of proangiogenic and antiangiogenic factors. PAR1, ADP, and GPVI stimulation favors proangiogenic, whereas PAR4 promotes antiangiogenic, factor release.

The platelet releasates from both PAR1- and PAR4-stimulated platelets enhanced EPC migration and tube formation, but had no influence on EPC proliferation. However, PAR1-PR–enhanced capillary network formation of EPCs was more profound, and the enhancement was even more evident in the mouse model of Matrigel implantation. The enhancements involve multiple factors, as intervention of either VEGF, SDF-1α, or MMP abolished platelet releasate–enhanced tube formation of EPCs.

Thrombin stimulation induces SDF-1a mRNA maturation, which leads to de novo synthesis of SDF-1α after activation. The newly synthesized SDF-1a may reinforce platelet angiogenic activities in remodelling and repair of the injured vessels.

Our findings support the notion that platelets are a versatile coordinator of angiogenesis.

7 FUTURE PERSPECTIVES

During the past decade, numerous data have advanced our knowledge and understanding of the role of EPCs in vascular regeneration and endothelial reparation. Still, many questions remain to be addressed.

EPCs have become a potential therapy in the treatment of cardiovascular diseases. However, it is still limited by several factors for clinical applications, such as cell senescence and differentiation during long time culture and functional impairment from patients with cardiovascular diseases. It should be of great interest to develop a new culture method to rescue EPCs from cellular senescence. Moreover, there is a need to improve EPC functions from the cardiovascular diseases patients for the further autologous transplantation.

Besides that, EPCs have some properties that are different from mature ECs, such as EPCs have higher proliferative capacity and more resistant against apoptosis under serum deprivation than mature ECs. Moreover, infusion of EPC, but not of mature ECs, promotes neovascularization after ischemia. So it could be a potent target to improve endothelia function if we can identify the major molecular by comparison the difference between EPC and EC.

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9 REFERENCES

1. Carmeliet, P., *Angiogenesis in life, disease and medicine.* Nature, 2005. **438**(7070): p. 932-6.
2. Risau, W., *Mechanisms of angiogenesis.* Nature, 1997. **386**(6626): p. 671-4.
3. Burri, P.H. and V. Djonov, *Intussusceptive angiogenesis--the alternative to capillary sprouting.* Mol Aspects Med, 2002. **23**(6S): p. S1-27.
4. Asahara, T., et al., *Isolation of putative progenitor endothelial cells for angiogenesis.* Science, 1997. **275**(5302): p. 964-7.
5. Bompais, H., et al., *Human endothelial cells derived from circulating progenitors display specific functional properties compared with mature vessel wall endothelial cells.* Blood, 2004. **103**(7): p. 2577-84.
6. Hur, J., et al., *Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis.* Arterioscler Thromb Vasc Biol, 2004. **24**(2): p. 288-93.
7. Kalka, C., et al., *Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization.* Proc Natl Acad Sci U S A, 2000. **97**(7): p. 3422-7.
8. Kawamoto, A., et al., *Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia.* Circulation, 2001. **103**(5): p. 634-7.
9. Andrews, R.K., et al., *Platelet interactions in thrombosis.* IUBMB Life, 2004. **56**(1): p. 13-8.
10. Shattil, S.J. and P.J. Newman, *Integrins: dynamic scaffolds for adhesion and signaling in platelets.* Blood, 2004. **104**(6): p. 1606-15.
11. Gawaz, M., H. Langer, and A.E. May, *Platelets in inflammation and atherogenesis.* J Clin Invest, 2005. **115**(12): p. 3378-84.
12. Roukis, T.S., T. Zgonis, and B. Tiernan, *Autologous platelet-rich plasma for wound and osseous healing: a review of the literature and commercially available products.* Adv Ther, 2006. **23**(2): p. 218-37.
13. Sabrkhany, S., A.W. Griffioen, and M.G. Oude Egbrink, *The role of blood platelets in tumor angiogenesis.* Biochim Biophys Acta, 2011. **1815**(2): p. 189-96.
14. Packham, M.A., *Role of platelets in thrombosis and hemostasis.* Can J Physiol Pharmacol, 1994. **72**(3): p. 278-84.
15. Rendu, F. and B. Brohard-Bohn, *The platelet release reaction: granules' constituents, secretion and functions.* Platelets, 2001. **12**(5): p. 261-73.
16. King, S.M. and G.L. Reed, *Development of platelet secretory granules.* Semin Cell Dev Biol, 2002. **13**(4): p. 293-302.
17. Patzelt, J. and H.F. Langer, *Platelets in angiogenesis.* Curr Vasc Pharmacol, 2012. **10**(5): p. 570-7.
18. McNicol, A. and S.J. Israels, *Platelet dense granules: structure, function and implications for haemostasis.* Thromb Res, 1999. **95**(1): p. 1-18.
19. Gerrard, J.M., J.G. White, and D.A. Peterson, *The platelet dense tubular system: its relationship to prostaglandin synthesis and calcium flux.* Thromb Haemost, 1978. **40**(2): p. 224-31.
20. Bennett, J.S., B.W. Berger, and P.C. Billings, *The structure and function of platelet integrins.* J Thromb Haemost, 2009. **7 Suppl 1**: p. 200-5.
21. Ginsberg, M.H., et al., *Platelet integrins.* Thromb Haemost, 1995. **74**(1): p. 352-9.
22. Rivera, J., et al., *Platelet GP Ib/IX/V complex: physiological role.* J Physiol Biochem, 2000. **56**(4): p. 355-65.
23. Rivera, J., et al., *Platelet receptors and signaling in the dynamics of thrombus formation.* Haematologica, 2009. **94**(5): p. 700-11.

24. Li, Z., et al., *Signaling during platelet adhesion and activation.* Arterioscler Thromb Vasc Biol, 2010. **30**(12): p. 2341-9.
25. Sarratt, K.L., et al., *GPVI and alpha2beta1 play independent critical roles during platelet adhesion and aggregate formation to collagen under flow.* Blood, 2005. **106**(4): p. 1268-77.
26. Arman, M. and K. Krauel, *Human platelet IgG Fc receptor FcgammaRIIA in immunity and thrombosis.* J Thromb Haemost, 2015.
27. Furie, B., B.C. Furie, and R. Flaumenhaft, *A journey with platelet P-selectin: the molecular basis of granule secretion, signalling and cell adhesion.* Thromb Haemost, 2001. **86**(1): p. 214-21.
28. Osada, M., et al., *Platelet activation receptor CLEC-2 regulates blood/lymphatic vessel separation by inhibiting proliferation, migration, and tube formation of lymphatic endothelial cells.* J Biol Chem, 2012. **287**(26): p. 22241-52.
29. Tomlinson, M.G., *Platelet tetraspanins: small but interesting.* J Thromb Haemost, 2009. **7**(12): p. 2070-3.
30. Goschnick, M.W. and D.E. Jackson, *Tetraspanins-structural and signalling scaffolds that regulate platelet function.* Mini Rev Med Chem, 2007. **7**(12): p. 1248-54.
31. Kahn, M.L., et al., *A dual thrombin receptor system for platelet activation.* Nature, 1998. **394**(6694): p. 690-4.
32. Charo, I.F., R.D. Feinman, and T.C. Detwiler, *Interrelations of platelet aggregation and secretion.* J Clin Invest, 1977. **60**(4): p. 866-73.
33. Hourani, S.M. and N.J. Cusack, *Pharmacological receptors on blood platelets.* Pharmacol Rev, 1991. **43**(3): p. 243-98.
34. Paul, B.Z., J. Jin, and S.P. Kunapuli, *Molecular mechanism of thromboxane A(2)-induced platelet aggregation. Essential role for p2t(ac) and alpha(2a) receptors.* J Biol Chem, 1999. **274**(41): p. 29108-14.
35. Trumel, C., et al., *A key role of adenosine diphosphate in the irreversible platelet aggregation induced by the PAR1-activating peptide through the late activation of phosphoinositide 3-kinase.* Blood, 1999. **94**(12): p. 4156-65.
36. Falker, K., D. Lange, and P. Presek, *ADP secretion and subsequent P2Y12 receptor signalling play a crucial role in thrombin-induced ERK2 activation in human platelets.* Thromb Haemost, 2004. **92**(1): p. 114-23.
37. Hechler, B., M. Cattaneo, and C. Gachet, *The P2 receptors in platelet function.* Semin Thromb Hemost, 2005. **31**(2): p. 150-61.
38. Gachet, C., et al., *Reversible translocation of phosphoinositide 3-kinase to the cytoskeleton of ADP-aggregated human platelets occurs independently of Rho A and without synthesis of phosphatidylinositol (3,4)-bisphosphate.* J Biol Chem, 1997. **272**(8): p. 4850-4.
39. Li, N., et al., *Effects of serotonin on platelet activation in whole blood.* Blood Coagul Fibrinolysis, 1997. **8**(8): p. 517-23.
40. Lopez-Vilchez, I., et al., *Serotonin enhances platelet procoagulant properties and their activation induced during platelet tissue factor uptake.* Cardiovasc Res, 2009. **84**(2): p. 309-16.
41. Ruggeri, Z.M., *Mechanisms initiating platelet thrombus formation.* Thromb Haemost, 1997. **78**(1): p. 611-6.
42. Savage, B., F. Almus-Jacobs, and Z.M. Ruggeri, *Specific synergy of multiple substrate-receptor interactions in platelet thrombus formation under flow.* Cell, 1998. **94**(5): p. 657-66.
43. Savage, B., E. Saldivar, and Z.M. Ruggeri, *Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor.* Cell, 1996. **84**(2): p. 289-97.

44. Frojmovic, M.M., *Platelet aggregation in flow: differential roles for adhesive receptors and ligands.* Am Heart J, 1998. **135**(5 Pt 2 Su): p. S119-31.
45. Ruggeri, Z.M., *Old concepts and new developments in the study of platelet aggregation.* J Clin Invest, 2000. **105**(6): p. 699-701.
46. Lopez, J.A., et al., *Bernard-Soulier syndrome.* Blood, 1998. **91**(12): p. 4397-418.
47. Sakariassen, K.S., P.A. Bolhuis, and J.J. Sixma, *Human blood platelet adhesion to artery subendothelium is mediated by factor VIII-Von Willebrand factor bound to the subendothelium.* Nature, 1979. **279**(5714): p. 636-8.
48. Tsuji, S., et al., *Real-time analysis of mural thrombus formation in various platelet aggregation disorders: distinct shear-dependent roles of platelet receptors and adhesive proteins under flow.* Blood, 1999. **94**(3): p. 968-75.
49. Coller, B.S., *The effects of ristocetin and von Willebrand factor on platelet electrophoretic mobility.* J Clin Invest, 1978. **61**(5): p. 1168-75.
50. Erhardt, J.A., et al., *P2X1 stimulation promotes thrombin receptor-mediated platelet aggregation.* J Thromb Haemost, 2006. **4**(4): p. 882-90.
51. Oury, C., et al., *Overexpression of the platelet P2X1 ion channel in transgenic mice generates a novel prothrombotic phenotype.* Blood, 2003. **101**(10): p. 3969-76.
52. Oury, C., et al., *P2X(1)-mediated activation of extracellular signal-regulated kinase 2 contributes to platelet secretion and aggregation induced by collagen.* Blood, 2002. **100**(7): p. 2499-505.
53. Huang, Z., et al., *P2X1-initiated p38 signalling enhances thromboxane A2-induced platelet secretion and aggregation.* Thromb Haemost, 2014. **112**(1): p. 142-50.
54. Herzog, B.H., et al., *Podoplanin maintains high endothelial venule integrity by interacting with platelet CLEC-2.* Nature, 2013. **502**(7469): p. 105-9.
55. Ni, H., et al., *Persistence of platelet thrombus formation in arterioles of mice lacking both von Willebrand factor and fibrinogen.* J Clin Invest, 2000. **106**(3): p. 385-92.
56. Ruggeri, Z.M., *Structure of von Willebrand factor and its function in platelet adhesion and thrombus formation.* Best Pract Res Clin Haematol, 2001. **14**(2): p. 257-79.
57. Nieswandt, B. and S.P. Watson, *Platelet-collagen interaction: is GPVI the central receptor?* Blood, 2003. **102**(2): p. 449-61.
58. Massberg, S., et al., *A crucial role of glycoprotein VI for platelet recruitment to the injured arterial wall in vivo.* J Exp Med, 2003. **197**(1): p. 41-9.
59. Plow, E.F., et al., *Ligand binding to integrins.* J Biol Chem, 2000. **275**(29): p. 21785-8.
60. Bennett, J.S., *Structure and function of the platelet integrin alphaIIbbeta3.* J Clin Invest, 2005. **115**(12): p. 3363-9.
61. Bledzka, K., S.S. Smyth, and E.F. Plow, *Integrin alphaIIbbeta3: from discovery to efficacious therapeutic target.* Circ Res, 2013. **112**(8): p. 1189-200.
62. Ma, L., et al., *Proteinase-activated receptors 1 and 4 counter-regulate endostatin and VEGF release from human platelets.* Proc Natl Acad Sci U S A, 2005. **102**(1): p. 216-20.
63. Italiano, J.E., Jr., et al., *Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released.* Blood, 2008. **111**(3): p. 1227-33.
64. Kamykowski, J., et al., *Quantitative immunofluorescence mapping reveals little functional coclustering of proteins within platelet alpha-granules.* Blood, 2011. **118**(5): p. 1370-3.
65. Jonnalagadda, D., L.T. Izu, and S.W. Whiteheart, *Platelet secretion is kinetically heterogeneous in an agonist-responsive manner.* Blood, 2012. **120**(26): p. 5209-16.
66. Battinelli, E.M., B.A. Markens, and J.E. Italiano, Jr., *Release of angiogenesis regulatory proteins from platelet alpha granules: modulation of physiologic and pathologic angiogenesis.* Blood, 2011. **118**(5): p. 1359-69.

67. Nicosia, R.F., S.V. Nicosia, and M. Smith, *Vascular endothelial growth factor, platelet-derived growth factor, and insulin-like growth factor-1 promote rat aortic angiogenesis in vitro.* Am J Pathol, 1994. **145**(5): p. 1023-9.
68. Pintucci, G., et al., *Trophic effects of platelets on cultured endothelial cells are mediated by platelet-associated fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF).* Thromb Haemost, 2002. **88**(5): p. 834-42.
69. Brill, A., H. Elinav, and D. Varon, *Differential role of platelet granular mediators in angiogenesis.* Cardiovasc Res, 2004. **63**(2): p. 226-35.
70. Brill, A., et al., *Platelet-derived microparticles induce angiogenesis and stimulate post-ischemic revascularization.* Cardiovasc Res, 2005. **67**(1): p. 30-8.
71. Bar, R.S., et al., *The effects of platelet-derived growth factor in cultured microvessel endothelial cells.* Endocrinology, 1989. **124**(4): p. 1841-8.
72. Pukac, L., J. Huangpu, and M.J. Karnovsky, *Platelet-derived growth factor-BB, insulin-like growth factor-I, and phorbol ester activate different signaling pathways for stimulation of vascular smooth muscle cell migration.* Exp Cell Res, 1998. **242**(2): p. 548-60.
73. Stellos, K., et al., *Platelet-derived stromal cell-derived factor-1 regulates adhesion and promotes differentiation of human CD34+ cells to endothelial progenitor cells.* Circulation, 2008. **117**(2): p. 206-15.
74. Stellos, K. and M. Gawaz, *Platelets and stromal cell-derived factor-1 in progenitor cell recruitment.* Semin Thromb Hemost, 2007. **33**(2): p. 159-64.
75. Rafii, S., et al., *Platelet-derived SDF-1 primes the pulmonary capillary vascular niche to drive lung alveolar regeneration.* Nat Cell Biol, 2015. **17**(2): p. 123-36.
76. Daub, K., et al., *Platelets induce differentiation of human CD34+ progenitor cells into foam cells and endothelial cells.* FASEB J, 2006. **20**(14): p. 2559-61.
77. Massberg, S., et al., *Platelets secrete stromal cell-derived factor 1alpha and recruit bone marrow-derived progenitor cells to arterial thrombi in vivo.* J Exp Med, 2006. **203**(5): p. 1221-33.
78. Kandler, B., et al., *Platelet-released supernatant increases matrix metalloproteinase-2 production, migration, proliferation, and tube formation of human umbilical vascular endothelial cells.* J Periodontol, 2004. **75**(9): p. 1255-61.
79. Langlois, S., D. Gingras, and R. Beliveau, *Membrane type 1-matrix metalloproteinase (MT1-MMP) cooperates with sphingosine 1-phosphate to induce endothelial cell migration and morphogenic differentiation.* Blood, 2004. **103**(8): p. 3020-8.
80. Lee, O.H., et al., *Sphingosine 1-phosphate induces angiogenesis: its angiogenic action and signaling mechanism in human umbilical vein endothelial cells.* Biochem Biophys Res Commun, 1999. **264**(3): p. 743-50.
81. English, D., et al., *Sphingosine 1-phosphate released from platelets during clotting accounts for the potent endothelial cell chemotactic activity of blood serum and provides a novel link between hemostasis and angiogenesis.* FASEB J, 2000. **14**(14): p. 2255-65.
82. Yatomi, Y., et al., *Sphingosine 1-phosphate as a major bioactive lysophospholipid that is released from platelets and interacts with endothelial cells.* Blood, 2000. **96**(10): p. 3431-8.
83. Brown, N.S., et al., *Thymidine phosphorylase induces carcinoma cell oxidative stress and promotes secretion of angiogenic factors.* Cancer Res, 2000. **60**(22): p. 6298-302.
84. Moghaddam, A., et al., *Thymidine phosphorylase is angiogenic and promotes tumor growth.* Proc Natl Acad Sci U S A, 1995. **92**(4): p. 998-1002.
85. Sengupta, S., et al., *Thymidine phosphorylase induces angiogenesis in vivo and in vitro: an evaluation of possible mechanisms.* Br J Pharmacol, 2003. **139**(2): p. 219-31.

86. Dobbs, S.P., et al., *Platelet-derived endothelial cell growth factor expression and angiogenesis in cervical intraepithelial neoplasia and squamous cell carcinoma of the cervix.* Ann Diagn Pathol, 2000. **4**(5): p. 286-92.
87. Takahashi, Y., et al., *Significance of platelet-derived endothelial cell growth factor in the angiogenesis of human gastric cancer.* Clin Cancer Res, 1998. **4**(2): p. 429-34.
88. Ishikawa, F., et al., *Identification of angiogenic activity and the cloning and expression of platelet-derived endothelial cell growth factor.* Nature, 1989. **338**(6216): p. 557-62.
89. Bagavandoss, P. and J.W. Wilks, *Specific inhibition of endothelial cell proliferation by thrombospondin.* Biochem Biophys Res Commun, 1990. **170**(2): p. 867-72.
90. Taraboletti, G., et al., *Platelet thrombospondin modulates endothelial cell adhesion, motility, and growth: a potential angiogenesis regulatory factor.* J Cell Biol, 1990. **111**(2): p. 765-72.
91. Luster, A.D., S.M. Greenberg, and P. Leder, *The IP-10 chemokine binds to a specific cell surface heparan sulfate site shared with platelet factor 4 and inhibits endothelial cell proliferation.* J Exp Med, 1995. **182**(1): p. 219-31.
92. Busch, C., et al., *Binding of platelet factor 4 to cultured human umbilical vein endothelial cells.* Thromb Res, 1980. **19**(1-2): p. 129-37.
93. Gentilini, G., et al., *Inhibition of human umbilical vein endothelial cell proliferation by the CXC chemokine, platelet factor 4 (PF4), is associated with impaired downregulation of p21(Cip1/WAF1).* Blood, 1999. **93**(1): p. 25-33.
94. Maione, T.E., et al., *Inhibition of angiogenesis by recombinant human platelet factor-4 and related peptides.* Science, 1990. **247**(4938): p. 77-9.
95. Jurasz, P., et al., *Generation and role of angiostatin in human platelets.* Blood, 2003. **102**(9): p. 3217-23.
96. Lucas, R., et al., *Multiple forms of angiostatin induce apoptosis in endothelial cells.* Blood, 1998. **92**(12): p. 4730-41.
97. Dhanabal, M., et al., *Endostatin induces endothelial cell apoptosis.* J Biol Chem, 1999. **274**(17): p. 11721-6.
98. O'Reilly, M.S., et al., *Endostatin: an endogenous inhibitor of angiogenesis and tumor growth.* Cell, 1997. **88**(2): p. 277-85.
99. Ma, L., et al., *Platelets modulate gastric ulcer healing: role of endostatin and vascular endothelial growth factor release.* Proc Natl Acad Sci U S A, 2001. **98**(11): p. 6470-5.
100. Murphy, A.N., E.J. Unsworth, and W.G. Stetler-Stevenson, *Tissue inhibitor of metalloproteinases-2 inhibits bFGF-induced human microvascular endothelial cell proliferation.* J Cell Physiol, 1993. **157**(2): p. 351-8.
101. Schnaper, H.W., et al., *Type IV collagenase(s) and TIMPs modulate endothelial cell morphogenesis in vitro.* J Cell Physiol, 1993. **156**(2): p. 235-46.
102. Radomski, A., et al., *Identification, regulation and role of tissue inhibitor of metalloproteinases-4 (TIMP-4) in human platelets.* Br J Pharmacol, 2002. **137**(8): p. 1330-8.
103. Heemskerk, J.W., E.M. Bevers, and T. Lindhout, *Platelet activation and blood coagulation.* Thromb Haemost, 2002. **88**(2): p. 186-93.
104. Schubert, P. and D.V. Devine, *De novo protein synthesis in mature platelets: a consideration for transfusion medicine.* Vox Sang, 2010. **99**(2): p. 112-22.
105. Rondina, M.T., et al., *The septic milieu triggers expression of spliced tissue factor mRNA in human platelets.* J Thromb Haemost, 2011. **9**(4): p. 748-58.
106. Denis, M.M., et al., *Escaping the nuclear confines: signal-dependent pre-mRNA splicing in anucleate platelets.* Cell, 2005. **122**(3): p. 379-91.
107. Lindemann, S., et al., *Activated platelets mediate inflammatory signaling by regulated interleukin 1beta synthesis.* J Cell Biol, 2001. **154**(3): p. 485-90.

108. Panes, O., et al., *Human platelets synthesize and express functional tissue factor.* Blood, 2007. **109**(12): p. 5242-50.
109. Deanfield, J.E., J.P. Halcox, and T.J. Rabelink, *Endothelial function and dysfunction: testing and clinical relevance.* Circulation, 2007. **115**(10): p. 1285-95.
110. Chiu, J.J. and S. Chien, *Effects of disturbed flow on vascular endothelium: pathophysiological basis and clinical perspectives.* Physiol Rev, 2011. **91**(1): p. 327-87.
111. Hadi, H.A., C.S. Carr, and J. Al Suwaidi, *Endothelial dysfunction: cardiovascular risk factors, therapy, and outcome.* Vasc Health Risk Manag, 2005. **1**(3): p. 183-98.
112. Rajendran, P., et al., *The vascular endothelium and human diseases.* Int J Biol Sci, 2013. **9**(10): p. 1057-69.
113. Ingram, D.A., et al., *Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood.* Blood, 2004. **104**(9): p. 2752-60.
114. Vasa, M., et al., *Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease.* Circ Res, 2001. **89**(1): p. E1-7.
115. Hill, J.M., et al., *Circulating endothelial progenitor cells, vascular function, and cardiovascular risk.* N Engl J Med, 2003. **348**(7): p. 593-600.
116. Werner, N., et al., *Circulating endothelial progenitor cells and cardiovascular outcomes.* N Engl J Med, 2005. **353**(10): p. 999-1007.
117. Werner, N. and G. Nickenig, *Endothelial progenitor cells in health and atherosclerotic disease.* Ann Med, 2007. **39**(2): p. 82-90.
118. Dimmeler, S., et al., *HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway.* J Clin Invest, 2001. **108**(3): p. 391-7.
119. Rehman, J., et al., *Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors.* Circulation, 2003. **107**(8): p. 1164-9.
120. Yoon, C.H., et al., *Synergistic neovascularization by mixed transplantation of early endothelial progenitor cells and late outgrowth endothelial cells: the role of angiogenic cytokines and matrix metalloproteinases.* Circulation, 2005. **112**(11): p. 1618-27.
121. Sharpe, E.E., 3rd, et al., *The origin and in vivo significance of murine and human culture-expanded endothelial progenitor cells.* Am J Pathol, 2006. **168**(5): p. 1710-21.
122. Lin, Y., et al., *Origins of circulating endothelial cells and endothelial outgrowth from blood.* J Clin Invest, 2000. **105**(1): p. 71-7.
123. Yoder, M.C., et al., *Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals.* Blood, 2007. **109**(5): p. 1801-9.
124. Nabel, E.G. and E. Braunwald, *A tale of coronary artery disease and myocardial infarction.* N Engl J Med, 2012. **366**(1): p. 54-63.
125. Strehlow, K., et al., *Estrogen increases bone marrow-derived endothelial progenitor cell production and diminishes neointima formation.* Circulation, 2003. **107**(24): p. 3059-65.
126. Krishnamurthy, P., et al., *Interleukin-10 deficiency impairs bone marrow-derived endothelial progenitor cell survival and function in ischemic myocardium.* Circ Res, 2011. **109**(11): p. 1280-9.
127. Suh, W., et al., *Transplantation of endothelial progenitor cells accelerates dermal wound healing with increased recruitment of monocytes/macrophages and neovascularization.* Stem Cells, 2005. **23**(10): p. 1571-8.
128. Marrotte, E.J., et al., *Manganese superoxide dismutase expression in endothelial progenitor cells accelerates wound healing in diabetic mice.* J Clin Invest, 2010. **120**(12): p. 4207-19.
129. Fadini, G.P., et al., *Endothelial progenitor cells in the natural history of atherosclerosis.* Atherosclerosis, 2007. **194**(1): p. 46-54.

130. Schmidt-Lucke, C., et al., *Reduced number of circulating endothelial progenitor cells predicts future cardiovascular events: proof of concept for the clinical importance of endogenous vascular repair.* Circulation, 2005. **111**(22): p. 2981-7.
131. Seeger, F.H., et al., *p38 mitogen-activated protein kinase downregulates endothelial progenitor cells.* Circulation, 2005. **111**(9): p. 1184-91.
132. Urbich, C., et al., *Cathepsin L is required for endothelial progenitor cell-induced neovascularization.* Nat Med, 2005. **11**(2): p. 206-13.
133. Walter, D.H., et al., *Impaired CXCR4 signaling contributes to the reduced neovascularization capacity of endothelial progenitor cells from patients with coronary artery disease.* Circ Res, 2005. **97**(11): p. 1142-51.
134. Lee, J.H., et al., *The Sulfated Polysaccharide Fucoidan Rescues Senescence of Endothelial Colony Forming Cells for Ischemic Repair.* Stem Cells, 2015.
135. Kocher, A.A., et al., *Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function.* Nat Med, 2001. **7**(4): p. 430-6.
136. Doyle, B., et al., *Progenitor cell therapy in a porcine acute myocardial infarction model induces cardiac hypertrophy, mediated by paracrine secretion of cardiotrophic factors including TGFbeta1.* Stem Cells Dev, 2008. **17**(5): p. 941-51.
137. Leistner, D.M., et al., *Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI): final 5-year results suggest long-term safety and efficacy.* Clin Res Cardiol, 2011. **100**(10): p. 925-34.
138. Schachinger, V., et al., *Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction: final one-year results of the TOPCARE-AMI Trial.* J Am Coll Cardiol, 2004. **44**(8): p. 1690-9.
139. Assmus, B., et al., *Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI).* Circulation, 2002. **106**(24): p. 3009-17.
140. Assmus, B., et al., *Long-term clinical outcome after intracoronary application of bone marrow-derived mononuclear cells for acute myocardial infarction: migratory capacity of administered cells determines event-free survival.* Eur Heart J, 2014. **35**(19): p. 1275-83.
141. Assmus, B., et al., *Clinical outcome 2 years after intracoronary administration of bone marrow-derived progenitor cells in acute myocardial infarction.* Circ Heart Fail, 2010. **3**(1): p. 89-96.
142. Schachinger, V., et al., *Intracoronary infusion of bone marrow-derived mononuclear cells abrogates adverse left ventricular remodelling post-acute myocardial infarction: insights from the reinfusion of enriched progenitor cells and infarct remodelling in acute myocardial infarction (REPAIR-AMI) trial.* Eur J Heart Fail, 2009. **11**(10): p. 973-9.
143. Dill, T., et al., *Intracoronary administration of bone marrow-derived progenitor cells improves left ventricular function in patients at risk for adverse remodeling after acute ST-segment elevation myocardial infarction: results of the Reinfusion of Enriched Progenitor cells And Infarct Remodeling in Acute Myocardial Infarction study (REPAIR-AMI) cardiac magnetic resonance imaging substudy.* Am Heart J, 2009. **157**(3): p. 541-7.
144. Schachinger, V., et al., *Improved clinical outcome after intracoronary administration of bone-marrow-derived progenitor cells in acute myocardial infarction: final 1-year results of the REPAIR-AMI trial.* Eur Heart J, 2006. **27**(23): p. 2775-83.
145. Schachinger, V., et al., *Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction.* N Engl J Med, 2006. **355**(12): p. 1210-21.
146. Dimmeler, S., et al., *Translational strategies and challenges in regenerative medicine.* Nat Med, 2014. **20**(8): p. 814-21.
147. Tepper, O.M., et al., *Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures.* Circulation, 2002. **106**(22): p. 2781-6.

148. Edelberg, J.M., et al., *Young adult bone marrow-derived endothelial precursor cells restore aging-impaired cardiac angiogenic function.* Circ Res, 2002. **90**(10): p. E89-93.
149. Fadini, G.P., et al., *Circulating endothelial progenitor cells are reduced in peripheral vascular complications of type 2 diabetes mellitus.* J Am Coll Cardiol, 2005. **45**(9): p. 1449-57.
150. Heiss, C., et al., *Impaired progenitor cell activity in age-related endothelial dysfunction.* J Am Coll Cardiol, 2005. **45**(9): p. 1441-8.
151. Folkman, J., *Tumor angiogenesis: therapeutic implications.* N Engl J Med, 1971. **285**(21): p. 1182-6.
152. Ferrara, N., et al., *Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene.* Nature, 1996. **380**(6573): p. 439-42.
153. Patan, S., *Vasculogenesis and angiogenesis as mechanisms of vascular network formation, growth and remodeling.* J Neurooncol, 2000. **50**(1-2): p. 1-15.
154. Gerhardt, H., et al., *VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia.* J Cell Biol, 2003. **161**(6): p. 1163-77.
155. Takahashi, H. and M. Shibuya, *The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions.* Clin Sci (Lond), 2005. **109**(3): p. 227-41.
156. Senger, D.R., et al., *Purification and NH2-terminal amino acid sequence of guinea pig tumor-secreted vascular permeability factor.* Cancer Res, 1990. **50**(6): p. 1774-8.
157. Bellomo, D., et al., *Mice lacking the vascular endothelial growth factor-B gene (Vegfb) have smaller hearts, dysfunctional coronary vasculature, and impaired recovery from cardiac ischemia.* Circ Res, 2000. **86**(2): p. E29-35.
158. Aase, K., et al., *Vascular endothelial growth factor-B-deficient mice display an atrial conduction defect.* Circulation, 2001. **104**(3): p. 358-64.
159. Karkkainen, M.J., et al., *Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins.* Nat Immunol, 2004. **5**(1): p. 74-80.
160. Baldwin, M.E., et al., *Vascular endothelial growth factor D is dispensable for development of the lymphatic system.* Mol Cell Biol, 2005. **25**(6): p. 2441-9.
161. Krishnan, J., et al., *Differential in vivo and in vitro expression of vascular endothelial growth factor (VEGF)-C and VEGF-D in tumors and its relationship to lymphatic metastasis in immunocompetent rats.* Cancer Res, 2003. **63**(3): p. 713-22.
162. Marconcini, L., et al., *c-fos-induced growth factor/vascular endothelial growth factor D induces angiogenesis in vivo and in vitro.* Proc Natl Acad Sci U S A, 1999. **96**(17): p. 9671-6.
163. Cao, Y., et al., *Vascular endothelial growth factor C induces angiogenesis in vivo.* Proc Natl Acad Sci U S A, 1998. **95**(24): p. 14389-94.
164. Carmeliet, P., et al., *Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions.* Nat Med, 2001. **7**(5): p. 575-83.
165. Tammela, T., et al., *Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation.* Nature, 2008. **454**(7204): p. 656-60.
166. Goel, H.L. and A.M. Mercurio, *VEGF targets the tumour cell.* Nat Rev Cancer, 2013. **13**(12): p. 871-82.
167. Siekmann, A.F. and N.D. Lawson, *Notch signalling limits angiogenic cell behaviour in developing zebrafish arteries.* Nature, 2007. **445**(7129): p. 781-4.
168. Nilsson, I., et al., *VEGF receptor 2/-3 heterodimers detected in situ by proximity ligation on angiogenic sprouts.* EMBO J, 2010. **29**(8): p. 1377-88.
169. Presta, M., et al., *Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis.* Cytokine Growth Factor Rev, 2005. **16**(2): p. 159-78.

170. Poole, T.J., E.B. Finkelstein, and C.M. Cox, *The role of FGF and VEGF in angioblast induction and migration during vascular development.* Dev Dyn, 2001. **220**(1): p. 1-17.
171. Bottcher, R.T. and C. Niehrs, *Fibroblast growth factor signaling during early vertebrate development.* Endocr Rev, 2005. **26**(1): p. 63-77.
172. Ortega, S., et al., *Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2.* Proc Natl Acad Sci U S A, 1998. **95**(10): p. 5672-7.
173. Pond, A.C., et al., *Fibroblast growth factor receptor signaling dramatically accelerates tumorigenesis and enhances oncoprotein translation in the mouse mammary tumor virus-Wnt-1 mouse model of breast cancer.* Cancer Res, 2010. **70**(12): p. 4868-79.
174. Grose, R. and C. Dickson, *Fibroblast growth factor signaling in tumorigenesis.* Cytokine Growth Factor Rev, 2005. **16**(2): p. 179-86.
175. Kanda, S., et al., *Fibroblast growth factor receptor 1-induced differentiation of endothelial cell line established from tsA58 large T transgenic mice.* Cell Growth Differ, 1996. **7**(3): p. 383-95.
176. Nakamura, T., et al., *Signals via FGF receptor 2 regulate migration of endothelial cells.* Biochem Biophys Res Commun, 2001. **289**(4): p. 801-6.
177. Pepper, M.S., et al., *Upregulation of urokinase receptor expression on migrating endothelial cells.* J Cell Biol, 1993. **122**(3): p. 673-84.
178. Heldin, C.H. and B. Westermark, *Mechanism of action and in vivo role of platelet-derived growth factor.* Physiol Rev, 1999. **79**(4): p. 1283-316.
179. Hart, C.E. and D.F. Bowen-Pope, *Platelet-derived growth factor receptor: current views of the two-subunit model.* J Invest Dermatol, 1990. **94**(6 Suppl): p. 53S-57S.
180. Hellstrom, M., et al., *Lack of pericytes leads to endothelial hyperplasia and abnormal vascular morphogenesis.* J Cell Biol, 2001. **153**(3): p. 543-53.
181. Abramsson, A., et al., *Analysis of mural cell recruitment to tumor vessels.* Circulation, 2002. **105**(1): p. 112-7.
182. Kaminski, W.E., et al., *Basis of hematopoietic defects in platelet-derived growth factor (PDGF)-B and PDGF beta-receptor null mice.* Blood, 2001. **97**(7): p. 1990-8.
183. Oshimori, N. and E. Fuchs, *The harmonies played by TGF-beta in stem cell biology.* Cell Stem Cell, 2012. **11**(6): p. 751-64.
184. Dickson, M.C., et al., *Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice.* Development, 1995. **121**(6): p. 1845-54.
185. Larsson, J., et al., *Abnormal angiogenesis but intact hematopoietic potential in TGF-beta type I receptor-deficient mice.* EMBO J, 2001. **20**(7): p. 1663-73.
186. Oshima, M., H. Oshima, and M.M. Taketo, *TGF-beta receptor type II deficiency results in defects of yolk sac hematopoiesis and vasculogenesis.* Dev Biol, 1996. **179**(1): p. 297-302.
187. Gallione, C.J., et al., *Two common endoglin mutations in families with hereditary hemorrhagic telangiectasia in the Netherlands Antilles: evidence for a founder effect.* Hum Genet, 2000. **107**(1): p. 40-4.
188. Brusgaard, K., et al., *Mutations in endoglin and in activin receptor-like kinase 1 among Danish patients with hereditary haemorrhagic telangiectasia.* Clin Genet, 2004. **66**(6): p. 556-61.
189. Frater-Schroder, M., et al., *Transforming growth factor-beta inhibits endothelial cell proliferation.* Biochem Biophys Res Commun, 1986. **137**(1): p. 295-302.
190. Pollman, M.J., L. Naumovski, and G.H. Gibbons, *Vascular cell apoptosis: cell type-specific modulation by transforming growth factor-beta1 in endothelial cells versus smooth muscle cells.* Circulation, 1999. **99**(15): p. 2019-26.
191. Mandriota, S.J., P.A. Menoud, and M.S. Pepper, *Transforming growth factor beta 1 down-regulates vascular endothelial growth factor receptor 2/flk-1 expression in vascular endothelial cells.* J Biol Chem, 1996. **271**(19): p. 11500-5.

192. Leksa, V., et al., *TGF-beta-induced apoptosis in endothelial cells mediated by M6P/IGFII-R and mini-plasminogen.* J Cell Sci, 2005. **118**(Pt 19): p. 4577-86.
193. Yan, Q. and E.H. Sage, *Transforming growth factor-beta1 induces apoptotic cell death in cultured retinal endothelial cells but not pericytes: association with decreased expression of p21waf1/cip1.* J Cell Biochem, 1998. **70**(1): p. 70-83.
194. Evrard, S.M., et al., *The profibrotic cytokine transforming growth factor-beta1 increases endothelial progenitor cell angiogenic properties.* J Thromb Haemost, 2012. **10**(4): p. 670-9.
195. Fagiani, E. and G. Christofori, *Angiopoietins in angiogenesis.* Cancer Lett, 2013. **328**(1): p. 18-26.
196. Suri, C., et al., *Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis.* Cell, 1996. **87**(7): p. 1171-80.
197. Shyu, K.G., et al., *Direct intramuscular injection of plasmid DNA encoding angiopoietin-1 but not angiopoietin-2 augments revascularization in the rabbit ischemic hindlimb.* Circulation, 1998. **98**(19): p. 2081-7.
198. Suri, C., et al., *Increased vascularization in mice overexpressing angiopoietin-1.* Science, 1998. **282**(5388): p. 468-71.
199. Thurston, G., et al., *Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1.* Science, 1999. **286**(5449): p. 2511-4.
200. Koblizek, T.I., et al., *Angiopoietin-1 induces sprouting angiogenesis in vitro.* Curr Biol, 1998. **8**(9): p. 529-32.
201. Hayes, A.J., et al., *Angiopoietin-1 and its receptor Tie-2 participate in the regulation of capillary-like tubule formation and survival of endothelial cells.* Microvasc Res, 1999. **58**(3): p. 224-37.
202. Witzenbichler, B., et al., *Chemotactic properties of angiopoietin-1 and -2, ligands for the endothelial-specific receptor tyrosine kinase Tie2.* J Biol Chem, 1998. **273**(29): p. 18514-21.
203. Kim, I., et al., *Angiopoietin-1 induces endothelial cell sprouting through the activation of focal adhesion kinase and plasmin secretion.* Circ Res, 2000. **86**(9): p. 952-9.
204. Abdel-Malak, N.A., et al., *Early growth response-1 regulates angiopoietin-1-induced endothelial cell proliferation, migration, and differentiation.* Arterioscler Thromb Vasc Biol, 2009. **29**(2): p. 209-16.
205. Kwak, H.J., et al., *Angiopoietin-1 is an apoptosis survival factor for endothelial cells.* FEBS Lett, 1999. **448**(2-3): p. 249-53.
206. Papapetropoulos, A., et al., *Direct actions of angiopoietin-1 on human endothelium: evidence for network stabilization, cell survival, and interaction with other angiogenic growth factors.* Lab Invest, 1999. **79**(2): p. 213-23.
207. Kim, I., et al., *Angiopoietin-1 regulates endothelial cell survival through the phosphatidylinositol 3'-Kinase/Akt signal transduction pathway.* Circ Res, 2000. **86**(1): p. 24-9.
208. Kwak, H.J., et al., *Angiopoietin-1 inhibits irradiation- and mannitol-induced apoptosis in endothelial cells.* Circulation, 2000. **101**(19): p. 2317-24.
209. Maisonpierre, P.C., et al., *Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis.* Science, 1997. **277**(5322): p. 55-60.
210. Kim, I., et al., *Angiopoietin-2 at high concentration can enhance endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway.* Oncogene, 2000. **19**(39): p. 4549-52.
211. Teichert-Kuliszewska, K., et al., *Biological action of angiopoietin-2 in a fibrin matrix model of angiogenesis is associated with activation of Tie2.* Cardiovasc Res, 2001. **49**(3): p. 659-70.

212. Mochizuki, Y., et al., *Angiopoietin 2 stimulates migration and tube-like structure formation of murine brain capillary endothelial cells through c-Fes and c-Fyn.* J Cell Sci, 2002. **115**(Pt 1): p. 175-83.
213. Gale, N.W., et al., *Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by Angiopoietin-1.* Dev Cell, 2002. **3**(3): p. 411-23.
214. Valenzuela, D.M., et al., *Angiopoietins 3 and 4: diverging gene counterparts in mice and humans.* Proc Natl Acad Sci U S A, 1999. **96**(5): p. 1904-9.
215. Lee, H.J., et al., *Biological characterization of angiopoietin-3 and angiopoietin-4.* FASEB J, 2004. **18**(11): p. 1200-8.
216. Dar, A., O. Kollet, and T. Lapidot, *Mutual, reciprocal SDF-1/CXCR4 interactions between hematopoietic and bone marrow stromal cells regulate human stem cell migration and development in NOD/SCID chimeric mice.* Exp Hematol, 2006. **34**(8): p. 967-75.
217. Lawler, J.W., H.S. Slayter, and J.E. Coligan, *Isolation and characterization of a high molecular weight glycoprotein from human blood platelets.* J Biol Chem, 1978. **253**(23): p. 8609-16.
218. Good, D.J., et al., *A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin.* Proc Natl Acad Sci U S A, 1990. **87**(17): p. 6624-8.
219. Bloch, W., et al., *The angiogenesis inhibitor endostatin impairs blood vessel maturation during wound healing.* FASEB J, 2000. **14**(15): p. 2373-6.
220. Eder, J.P., Jr., et al., *Phase I clinical trial of recombinant human endostatin administered as a short intravenous infusion repeated daily.* J Clin Oncol, 2002. **20**(18): p. 3772-84.
221. Kulke, M.H., et al., *Phase II study of recombinant human endostatin in patients with advanced neuroendocrine tumors.* J Clin Oncol, 2006. **24**(22): p. 3555-61.
222. O'Reilly, M.S., *Angiostatin: an endogenous inhibitor of angiogenesis and of tumor growth.* EXS, 1997. **79**: p. 273-94.
223. Schmitz, V., et al., *Plasminogen derivatives encoding kringles 1-4 and kringles 1-5 exert indirect antiangiogenic and direct antitumoral effects in experimental lung cancer.* Cancer Invest, 2008. **26**(5): p. 464-70.
224. Ansell, P.J., et al., *Recombinant kringle 5 from plasminogen antagonises hepatocyte growth factor-mediated signalling.* Eur J Cancer, 2010. **46**(5): p. 966-73.
225. Gimbrone, M.A., Jr., et al., *Preservation of vascular integrity in organs perfused in vitro with a platelet-rich medium.* Nature, 1969. **222**(5188): p. 33-6.
226. Horstman, L.L. and Y.S. Ahn, *Platelet microparticles: a wide-angle perspective.* Crit Rev Oncol Hematol, 1999. **30**(2): p. 111-42.
227. Tan, K.T. and G.Y. Lip, *The potential role of platelet microparticles in atherosclerosis.* Thromb Haemost, 2005. **94**(3): p. 488-92.
228. Kim, H.K., et al., *Platelet microparticles induce angiogenesis in vitro.* Br J Haematol, 2004. **124**(3): p. 376-84.
229. Kim, H.K., et al., *Elevated levels of circulating platelet microparticles, VEGF, IL-6 and RANTES in patients with gastric cancer: possible role of a metastasis predictor.* Eur J Cancer, 2003. **39**(2): p. 184-91.
230. Laffont, B., et al., *Activated platelets can deliver mRNA regulatory Ago2*microRNA complexes to endothelial cells via microparticles.* Blood, 2013. **122**(2): p. 253-61.
231. Pipili-Synetos, E., E. Papadimitriou, and M.E. Maragoudakis, *Evidence that platelets promote tube formation by endothelial cells on matrigel.* Br J Pharmacol, 1998. **125**(6): p. 1252-7.
232. Marcondes, S., et al., *Platelets induce human umbilical vein endothelial cell proliferation through P-selectin.* Life Sci, 2000. **66**(19): p. 1817-26.

233. Sterk, L.M., et al., *Association of the tetraspanin CD151 with the laminin-binding integrins alpha3beta1, alpha6beta1, alpha6beta4 and alpha7beta1 in cells in culture and in vivo.* J Cell Sci, 2002. **115**(Pt 6): p. 1161-73.
234. Varga-Szabo, D., I. Pleines, and B. Nieswandt, *Cell adhesion mechanisms in platelets.* Arterioscler Thromb Vasc Biol, 2008. **28**(3): p. 403-12.
235. Schaff, M., et al., *Integrin alpha6beta1 is the main receptor for vascular laminins and plays a role in platelet adhesion, activation, and arterial thrombosis.* Circulation, 2013. **128**(5): p. 541-52.
236. Caiado, F. and S. Dias, *Endothelial progenitor cells and integrins: adhesive needs.* Fibrogenesis Tissue Repair, 2012. **5**: p. 4.
237. Kent, K.C., et al., *Platelet attachment stimulates endothelial cell regeneration after arterial injury.* Surgery, 1995. **117**(3): p. 276-81.
238. Etulain, J., et al., *Platelet-mediated angiogenesis is independent of VEGF and fully inhibited by aspirin.* Br J Pharmacol, 2013. **170**(2): p. 255-65.
239. Bouvard, C., et al., *alpha6-integrin subunit plays a major role in the proangiogenic properties of endothelial progenitor cells.* Arterioscler Thromb Vasc Biol, 2010. **30**(8): p. 1569-75.
240. van Nispen tot Pannerden, H., et al., *The platelet interior revisited: electron tomography reveals tubular alpha-granule subtypes.* Blood, 2010. **116**(7): p. 1147-56.
241. van Holten, T.C., et al., *Quantitative proteomics analysis reveals similar release profiles following specific PAR-1 or PAR-4 stimulation of platelets.* Cardiovasc Res, 2014. **103**(1): p. 140-6.
242. Etulain, J., et al., *Stimulation of PAR-1 or PAR-4 promotes similar pattern of VEGF and endostatin release and pro-angiogenic responses mediated by human platelets.* Platelets, 2015: p. 1-6.
243. Rowley, J.W., H. Schwertz, and A.S. Weyrich, *Platelet mRNA: the meaning behind the message.* Curr Opin Hematol, 2012. **19**(5): p. 385-91.
244. Weyrich, A.S., et al., *Signal-dependent translation of a regulatory protein, Bcl-3, in activated human platelets.* Proc Natl Acad Sci U S A, 1998. **95**(10): p. 5556-61.
245. Huang, Z., et al., *Thrombin induces de novo protein synthesis of stromal cell-derived factor-1alpha but not angiostatin in human platelets.* J Thromb Haemost, 2012. **10**(10): p. 2202-5.